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INDIGENOUS ORGANIC MATTER ON THE MOON

By Carl Sagan

YERKES OBSERVATORY, UNIVERSITY OF CHICAGO, WILLIAMS BAY, WISCONSIN, AND PHYSICS RESEARCH DEPARTMENT, ARMOUR RESEARCH FOUNDATION, CHICAGO, ILLINOIS

Communicated by H. J. Muller, and read before the Academy, November 16, 1959

Studies of the early history of the solar system[1, 2] indicate that for some period after its formation the Moon possessed a reducing atmosphere of secondary origin, composed largely of CH_4, NH_3, and H_2O, with smaller amounts of H_2 and the interaction products of these molecules. The effect of solar ultraviolet light (and electric discharge) on such an atmosphere is well known; organic molecules of fair complexity—up to molecular weight $\sim$100—are produced almost independently of the relative proportions of precursors. Amino and other organic acids, pyrroles, pyridines, and simple hydrocarbons and their polymers are among the synthesized molecules.[3–5] The purpose of the present paper is to estimate the quantity of organic matter which formed in the primitive lunar atmosphere, diffused to the surface, and survived to the present day.[6]

Consider ultraviolet radiation of intensity Q photons cm^{-2} sec^{-1} in the synthetically effective wavelengths falling for t seconds on an opaque gaseous envelope surrounding the Moon, and producing molecules of mean molecular weight μ with over-all quantum yield ϕ. Neglecting lunar gravitational capture of molecules produced outside a cylinder of lunar radius extending from the Moon to the Sun, the mean surface density of synthesized material will be

$$\sigma = \frac{\phi Q \mu}{4 N_A} t \quad \text{gm cm}^{-2},$$

where N_A is Avogadro's number. We have assumed that ϕ is independent of wavelength in the synthetically effective region. The values adopted for ϕ, Q, and t are now discussed in turn.

Because the molecular weight of the synthesized molecules was greater than the mean molecular weight of the lunar atmosphere, these molecules must have diffused to the surface under the influence of the lunar gravitational field. The time for such molecules to diffuse to atmospheric depths which are opaque in the photodissociating ultraviolet can be shown to be less than the time between successive absorptions of photodissociating photons.[6] The reason is essentially that, for most important organic precursors, the long wavelength limit for photoproduction exceeds the long wavelength limit for photolysis. Aldehydes are the notable exceptions. Oxygen-containing molecules must have been formed primarily near the

lunar surface. Recently a series of experiments on ultraviolet synthesis of amino acids in an atmosphere similar to that of the early Moon has been performed by Groth.[7] The quantum yield is found to be approximately independent of wavelength between λ2537 and λ1470. Since the time between molecular collisions was much shorter than the time between successive absorptions of ultraviolet photons in the primitive lunar atmosphere, the difference in pressure, temperature, and density between early lunar and contemporary laboratory conditions should not affect the order of magnitude of the quantum yield. With these considerations in mind, we adopt from Groth's data an over-all quantum yield for the photoproduction of amino acids in the early lunar atmosphere of $\phi = 10^{-6}$, a conservative value probably uncertain by a factor of ten.

From recent models of the evolution of the Sun,[8-10] the ultraviolet black body temperatures and geometrical dilution factors can be computed, and the radiation flux shortward of a given wavelength obtained from an integration of the Planck distribution function, for any epoch in the past. At the time the Sun's evolutionary track in the Hertzsprung-Russell diagram joined the main sequence, about 5×10^9 years ago, the quiet solar ultraviolet photon flux at wavelengths shortward of λ2600 in the vicinity of the Moon, and with no absorption in interplanetary space, is computed to be $Q = 4 \times 10^{14}$ photons cm^{-2} sec^{-1}, about half the present flux.

If not replenished from the interior, a lunar atmosphere will escape to space in roughly 10^3 years, as can be computed from the work of Spitzer.[11] Hence the lifetime of the secondary lunar atmosphere depended entirely on the supply rate of gases from the lunar interior. This is very difficult to estimate, but it is not unlikely that extensive lunar outgassing maintained an atmosphere opaque to ultraviolet radiation for at least 10^7 or 10^8 years; it is important to note that although the lunar craters are probably not volcanic in nature, other lunar surface features exist which are of undoubted igneous origin.[12]

With $\phi = 10^{-6}$, $Q = 4 \times 10^{14}$ cm^{-2} sec^{-1}, $\mu = 100$, and $t = 3 \times 10^{14}$ sec, the derived amino acid surface density is then $\sigma \simeq 5$ gm. cm^{-2}. The result is of course very rough—the principal uncertainty being the value of t—but it indicates that quite considerable quantities of organic matter may have been deposited on the primitive Moon. Miller[4] and Groth[7] find efficient production of other substances beside amino acids, some with greater quantum yields (especially formic and acetic acids) and many with lesser quantum yields. During the time of deposition, the lunar atmosphere would have inhibited thermo- and photodissociation of the deposited molecules.

As the secondary lunar atmosphere escaped to space, and replenishment from the interior gradually fell off, the rate of atmospheric organic synthesis declined, and short wavelength radiation penetrated nearer to the surface. In addition, the surface temperature slowly rose, due both to the loss of the insulating atmosphere, and to radioactive heating. The effect of heat and ultraviolet light on the molecules cited above is most remarkable. Although the second law of thermodynamics is obeyed, a large fraction of the molecules, with activation energies supplied, partake in organic syntheses of a higher order of complexity. Polypeptides arise from amino acids, hydrocarbon dimers and trimers form long-chain polymers, and in general very complex organic molecules are constructed.[3, 5, 13] Finally, because complex molecules are more resistant to heat and radiation than are simpler mol-

ecules, the syntheses are biased toward the net production of the most complex organic molecules.[14, 15]

Although continued radiation and high temperature would lead to the eventual destruction of all these molecules, we must remember that meteoritic matter was falling into the lunar atmosphere throughout the period of organic synthesis. Whipple[16] estimates that about 50 gm cm^{-2} falls on the Moon each 10^8 years at present rates of infall. In addition, it is almost certain that the rate of meteoritic infall on the Moon in primitive times was greater than today. As a consequence, the Moon's surface must have received a dust cover, probably composed primarily of silicates and ices, which can be identified, at least in part, with the present lunar surface material. The organic molecules would then be covered by a protective layer, insulating them from the extremes of lunar temperature and absorbing the incident solar radiation and subsequent meteoritic infall. At an average temperature $< 0°C$ and only mild fluctuations, the thermostability half-lives of most organic molecules are of the order of the age of the solar system.[17] Provided that no large-scale destructive events have occurred subsequently, we may anticipate the presence of complex organic matter beneath the dust layer with a mean surface density of perhaps 10 gm cm^{-2}. With a time-constant rate of meteoritic infall, and no escape of surface material to space, the layer of organic matter would be localized at a depth of some tens of meters. These remarks apply properly only to regions which have had no extensive lava flows; the southern highland appears to be such a region, as does much of the far side of the Moon.

A sample of appropriate lunar subsurface material should then have an organic fraction easily detectable by simple chemical techniques. A qualitative and quantitative analysis would give important evidence concerning prebiological organic syntheses and the early history of the solar system.

The author is indebted to Drs. G. P. Kuiper, Joshua Lederberg, H. J. Muller, and L. Reiffel, and to Lynn Sagan, for suggestions and constructive criticism.

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[6] A more complete discussion will be published as a monograph by the Panel on Extraterrestrial Life, Armed Forces—National Research Council Committee on Bio-Astronautics, National Academy of Sciences. The production of organic molecules in the atmospheres of other bodies of the solar system will be discussed elsewhere.

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BIOLOGICAL CONTAMINATION OF THE MOON

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The extensive deposition of both hard- and soft-landing packages on the lunar surface seems to be imminent. There has been recent concern that terrestrial microorganisms and organic matter, deposited with the packages, may obscure detection of, or interact with, possible organisms or organic matter indigenous to the Moon.[1, 2] Such biological contamination of the Moon would represent an unparalleled scientific disaster, eliminating promising approaches to such problems as the early history of the solar system, the chemical composition of matter in the remote past, the origin of life on Earth, and the possibility of extraterrestrial life. Because of the Moon's unique situation as a large unweathered body at an intermediate distance from the Sun, scientific opportunities lost on the Moon may not be recoupable elsewhere.

There are four possible kinds of lunar biological contamination, which we discuss under the following headings:

1. *Biomixy.*—The Moon may contain no indigenous living organisms, and may be incapable of supporting terrestrial organisms. Nevertheless, there may be relics of primitive indigenous organisms and deposited cosmobiota on or beneath the surface. Especially on a low-gravity, high-vacuum body such as the Moon, a vehicle impacting at or near escape velocity will distribute its contents over most of the lunar surface. Subsequent investigations might then be unable to distinguish among primitive indigenous organisms, cosmobiota, and terrestrial microbiological contamination.

2. *Sapromixy.*—The Moon may contain no indigenous living organisms, and may be incapable of supporting terrestrial organisms. But subsurface prebiological organic matter may exist which would be indistinguishable from deposited terrestrial organic matter, either biological or abiological in origin.

3. *Phagomixy.*—The Moon may contain no indigenous living organisms, but may be capable of supporting some terrestrial organisms. This would require subsurface organic matter, moisture, and heat sources. The possibility then exists that a deposited terrestrial microorganism, in the absence of biological competitors or predators, will multiply at a geometric rate limited only by the availability of

water and metabolites. Such a biological explosion might in a short time destroy large quantities of organic matter produced in the early history of the Moon.

4. *Ecomixy.*—The Moon may contain indigenous living organisms. There is then the possibility that deposited terrestrial microorganisms, by competition with or parasitism upon even one species of lunar organism, will disrupt the autochthonous ecology completely.

The purpose of the present paper is to evaluate these possibilities.[3]

Survival of Terrestrial Microorganisms on the Moon.—There seem to be three major hazards for survival of terrestrial life on the Moon—temperature, corpuscular radiation, and solar electromagnetic radiation—which we discuss in turn. The probable absence of oxygen, water, and other substances from the Moon's surface is not, of course, evidence against survival, particularly of dormant anaerobic microorganisms; but it does preclude their reproduction on the surface of the Moon.

Surface temperatures range from about $+100°C$ to about $-180°C$ during a lunar day and night.[4] However, just beneath the surface the temperatures are much more moderate; at a depth of less than half a meter, microwave thermal radiation data indicate a temperature variation between $0°$ and $-70°C$.[5] It is important to note that the thermostability of microorganisms is greatly improved in the dried state and *in vacuo*, as they would be on the Moon; even at temperatures approaching 100°C, survival of a significant fraction of the total number of vegetative bacterial cells and spores may be expected.[6] Still higher temperatures are required to inactivate desoxyribonucleic acid.[7] Especially since it is likely that many of the deposited microorganisms will find themselves lodged just beneath the surface, as we discuss below, the debilitating effects of the high lunar surface temperatures can be neglected.

The work of Biermann on the acceleration of comet tails indicates a flux of solar protons in the vicinity of the Moon of about 5×10^{10} protons cm^{-2} sec^{-1}, and a mean particle kinetic energy of about 1 KeV.[8] Charged particles will be excluded from regions where the magnetic energy density exceeds the particle kinetic energy density. For the surface of the Moon, the lunar magnetic field strength must exceed about 10^{-2} gauss for the solar proton flux to be deflected.

From lunar occultations of cosmic radio sources, it can be estimated that the lunar atmosphere contains less than 10^{14} molecules above each square centimeter of surface.[9] Ultraviolet absorption cross sections for all molecules likely to be in the lunar atmosphere are generally less than 10^{-16} cm^2 at all wavelengths. Hence the optical depth in the ultraviolet is less than 10^{-2}, and there is no attenuation of incident solar ultraviolet radiation by the lunar atmosphere. For the solar proton wind, a 1 KeV proton has a range of about 10^{-2} cm-atm, or roughly 3×10^{17} molecules cm^{-2}. Consequently, if the lunar magnetic field strength is less than about 10^{-2} gauss, the solar proton stream strikes the Moon's surface with negligible loss of energy due to its passage through the tenuous lunar atmosphere. A similar conclusion applies to the more energetic cosmic rays.

We now consider the effect of these radiations on terrestrial microorganisms deposited on the lunar surface. Expressions can be derived for the time, t, in seconds, in which a population of N_0 organisms, having a mean lethal dose D for a given radiation, and characteristic diameter, a, in cm, is reduced to N organisms by radi-

ation of intensity I ergs cm^{-2} sec^{-1}. For ionizing radiation with D in reps, one finds[3] with an exponential survival law,

$$t = 214a\rho(D/I)\ [1 - e^{-(\mu/\rho)\rho a}]^{-1} \log_{10} (N_0/N),$$

where μ/ρ is the mass absorption coefficient of the organism in cm^2 gm^{-1}, and ρ is its density in gm cm^{-3}. When the mean lethal dose is given directly in units of ergs cm^{-2}, as is the case for nonionizing radiation, the expression for t is the same if the coefficient $214a\rho$ is replaced by 2.30.

The intensities to be used in this equation are those appropriate to the lunar surface for negligible atmosphere and magnetic field strength, and so are equally appropriate to interplanetary space in the vicinity of the Earth-Moon system. Consequently, the derived lifetimes are also those of an unprotected microorganism in free space, and so have a bearing on the panspermia or cosmobiota hypothesis.

For ionizing radiation, the high value of $D = 10^7$ rep was chosen.[3, 10] For ultraviolet radiation, a mean value $D = 10^7$ erg cm^{-2} was selected for 2000 Å $\leq \lambda \leq$ 3000 Å; for $\lambda \leq$ 2000 Å, $D < 10^6$ erg cm^{-2}.[3, 10] It should be emphasized that these mean lethal doses are purposely high to allow for anaerobiosis and drying. The resulting lifetimes should be upper limits, except, perhaps, where $\rho/\mu \ll \rho a$ for ionizing radiation.

A 1 kg instrumented lunar package may easily contain 10^{10} microorganisms;[1] it is very unlikely that any packages for the immediate future will contain as many as 10^{20} microorganisms. With these limits on N_0/N, we compute that all microorganisms deposited and exposed to the Sun will be killed by ultraviolet radiation in a few hours. Similarly, fully illuminated microorganisms in cislunar space will also survive only a few hours. Hence the panspermia hypothesis is untenable for unprotected microorganisms of comparable radiosensitivity to terrestrial microorganisms. On the other hand, suppose some microorganisms are deposited in a lunar crevasse or other depression, always shielded from solar radiation. Then, radiation killing will be effected only by cosmic rays and by natural radioactivity. Microorganisms shielded from the Sun, but just beneath the lunar surface, need, at the assumed high D, more than a few hundred million years to be killed by cosmic radiation; organisms at greater depths will have even longer survival times. Similarly, cosmobiota imbedded in, for example, a meteorite would have lifetimes comparable to the age of the solar system, and under these circumstances the panspermia hypothesis remains tenable.

From eclipse temperature measurements, and polarimetric and radio observations, it is known that a dust covering exists on the Moon, but estimates of its depth range from millimeters to miles. However, Whipple[11] has called attention to the experimental fact that dust, irradiated in a vacuum, will congeal, forming a low-density, semiporous matrix. If the lunar surface material has a similar structure, microorganisms can then be lodged in the interstices of the matrix, in such positions as to be shielded from the Sun's rays at all angles of incidence. Thus we may anticipate the survival for very great periods of time of perhaps a few per cent of those dormant anaerobic microorganisms deposited at the lunar surface. A determination of the microstructure of the Moon's surface is of great importance to corroborate this conclusion.

Survival of Deposited Nonliving Organic Matter on the Moon.—The killing of an organism, of course, need not involve a great deal of chemical dissociation, and long after death occurs, in an anhydrous aseptic environment, many aspects of the organism's characteristic biochemical structure will be maintained. Potential activity of cellular DNA may outlast the survival of the cell itself. After long periods of continued irradiation, enough bonds would be broken to destroy most of the long-chain biological polymers. The problem is complicated by the existence of radiation-protection devices (catalase, cytochromes, sulfhydryl compounds, photoreactivation mechanisms) in most contemporary organisms.

Because of the Franck–Rabinowitch cage effect, the collection of dissociated molecules arising from the original organism would tend to remain in close physical contact. Ionizing radiation is very much more efficient than nonionizing radiation in depolymerizing and dissociating organic molecules. Breaking of all hydrogen molecular bonds and charring occurs at about 10^{10} rep.[4] Charring by the solar proton wind of all but 10^{-15} of the exposed molecular aggregates occurs in from months to years, depending on the size of the dissociated organism. If, however, the lunar surface magnetic field exceeds 10^{-2} gauss and the proton wind does not penetrate to the surface, it may take as long as several hundred thousand years for charring to be induced by soft solar x-rays in the 50 Å region. Thus the value of the lunar magnetic field strength has great relevance for the question of possible biomictic and sapromictic contamination of the Moon. Preliminary data from Lunik II indicate a surface field of about 3×10^{-4} gauss;[12] in this case, solar protons will effect charring in the shorter time scale.

As dissociation advances, lunar temperature effects would become more important, small molecules being readily dissociated near 100°C. For example, the most thermostable amino acid, alanine, has a thermostability half-life at 100°C of approximately 10^3 years,[13] with most other amino acids having half-lives not less than ten years. Molecules shielded from radiative dissociation would be relatively unaffected by lunar temperatures and, if lodged beneath a few centimeters of insulating lunar surface material, would have lifetimes determined by the cosmic ray flux and natural radioactivity.

Conclusion.—We are now in a position to evaluate the four possible circumstances of lunar biological contamination described in the beginning of this paper. Assume that a 10^4 kg vehicle (the order of magnitude of existing unfueled final stage carrier rocket masses) with a microorganism population of 10^{10} per kg impacts the Moon at escape velocity. If half the impact energy is diverted to particle kinetic energy of the explosion products, about sixteen per cent of the microorganisms will be moving in an upward direction with velocity between the circular and the escape velocities after impact. Impact forces and temperatures are non-lethal because of the short deceleration time-scale ($\sim 10^{-3}$ secs).[3] Thus, about 1.6×10^{13} microorganisms will be deposited approximately uniformly over the lunar surface, the mean surface density being about 0.4 microorganisms per square meter. Near the impact area, the surface density of microorganisms will be considerably greater. We have calculated that all but the small fraction of deposited microorganisms which is shielded from solar illumination will be killed by ultraviolet radiation in hours. Therefore the mean surface density of viable microorganisms deposited by such a vehicle should be less than 0.01 m^{-2}.

This surface density of viable microorganisms is well below that detectable by existing biological techniques, such as plating; Lederberg[14] believes that existing techniques can be immediately extended to detect one microorganism m^{-2}, but considerable further refinement would be required to detect 10^{-2} m^{-2} where subsurface sample-gathering is also required. Cosmobiota and remnants of indigenous lunar organisms, if such exist, would be sequestered almost exclusively at much greater depths below the surface than would deposited terrestrial microorganisms. We conclude that the probability of biomictic contamination of the Moon is very low.

Since a typical bacterium has a mass of roughly 10^{-12} gm, the amount of organic matter deposited as microorganisms by impacting vehicles is completely negligible compared with the amount of indigenous organic matter which has probably survived from the early history of the Moon.[15] A similar conclusion follows for organic matter arising from vehicle structural elements. We conclude that the probability of sapromictic contamination is negligible.

An investigation of the production of organic molecules in the primitive lunar atmosphere[15] has indicated that as much as 10 gm cm^{-2} of organic matter may be buried beneath the present surface of the Moon at a depth crudely estimated at a few tens of meters. Subsurface temperatures are known to avoid the extremes of lunar surface temperatures,[5] and a mechanism has been proposed whereby biologically optimum temperatures may be provided by radioactive heating, and localized at some depth by a hydrostatic pressure-induced increase in the thermal conductivity of the dust.[16] At such temperatures and depths, moisture should be expected, arising from meteoritic and organic bound water. A viable terrestrial microorganism introduced into such an environment might reproduce very rapidly. The extent of phagomictic contamination would depend on the degree to which concentrations of organic matter are in mutual contact under the lunar surface, on possible self-limitation of the reproduction rate by accumulation of catabolites, and, of course, on the presence of the specific growth requirements for individual varieties of microorganisms. It is very improbable that a given organism deposited near the surface would find its way to a depth of tens of meters, but when 10^{14} microorganisms are deposited, the situation is very different. Although the presence of appropriate temperatures, moisture and organic matter for terrestrial microbiological multiplication remains to be demonstrated rigorously, at the present writing the likelihood of phagomictic contamination of the Moon is not negligible.

Because of its great potential importance, the admittedly very speculative possibility must be raised that life arose on the Moon before the secondary lunar atmosphere was lost to space. Conditions on the Moon 5×10^9 years ago were probably not very different from conditions on the Earth 5×10^9 years ago. Recent thinking on the origin of life on this planet is increasingly inclined toward a very rapid origin of the first self-reproducing system. If a similar event also occurred on the Moon, natural selection may be expected to have kept pace with the increasingly more severe lunar environment, at least for some period of time. If subsurface conditions exist similar to those described in the preceding paragraph, then the possibility of an extant lunar parabiology must not be dismissed in as cavalier a manner as it has been in the past. Even if indigenous lunar organisms exist, the occurrence of ecomictic contamination will depend on such matters as the stereo-

chemistry and detailed ecology of the autochthons; but in our present ignorance the possibility of ecomixy cannot be excluded. Of all the kinds of biological contamination, this would represent the greatest loss.

To date, two man-made objects have impacted the Moon, the instrument package and the 7,700 kg carrier rocket of Lunik II. According to reports from the Soviet Union, both were sterilized.[17] It is recommended that all future lunar probes be scrupulously decontaminated.

Summary.—The probability of survival of a terrestrial microorganism, accidentally deposited on the Moon by an impacting lunar probe, has been computed. A population of the least radiosensitive dormant anaerobic microorganisms, if exposed to solar ultraviolet radiation, would be completely killed in hours. The resulting organic dissociation products would remain intact for much longer periods of time—0.1 to 10 years if the lunar surface magnetic field strength is much less than 10^{-2} gauss (so incident solar protons are magnetically deflected), and 10^4 to 10^5 years if the field strength exceeds 10^{-2} gauss. Organisms shielded from solar illumination, perhaps in congealed dust matrix interstices, might survive cosmic radiation for 10^9 years or more; lunar subsurface temperatures are too low to impede survival. The possible circumstances of lunar biological contamination are then discussed. It is concluded that the probability is very low that deposited terrestrial microorganisms and organic matter will be confused with indigenous lunar organisms or organic matter; but that the explosive reproduction of terrestrial microorganisms in indigenous lunar organic matter, and the disruption of the ecologies of hypothetical lunar organisms are remote but nonnegligible possibilities.

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*INTRAMOLECULAR MODELS DEPICTING THE KINETIC IMPORTANCE OF "FIT" IN ENZYMATIC CATALYSIS**

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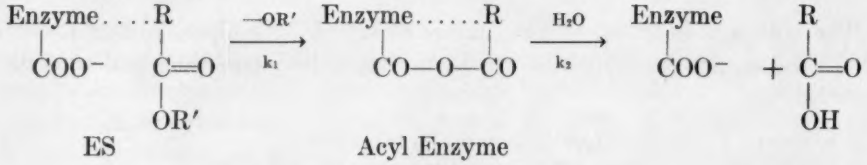
Communicated by Albert L. Lehninger, February 25, 1960

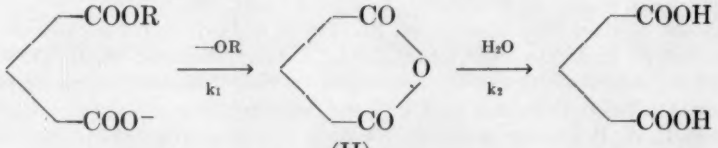
This communication describes a few of the intramolecular models which have been studied in this laboratory in order to ascertain the kinetic effect of steric compression on an intramolecular catalysis of the hydrolysis of an ester bond. In enzyme catalysis the bond making and breaking processes occur within a complex of enzyme and substrate (ES) presumably *via* the participation of particular functional groups at the "active site." For the case of certain esteratic enzymes an intracomplex nucleophilic displacement of —OR′ from R − CO − OR′ to give an acyl enzyme as an intermediate is involved. Thus, for the enzyme ficin, a carboxyl anion has been suggested to be the intracomplex participant.[1] The reaction may be pictured schematically as (I):



ES Acyl Enzyme

(I)

The similarity between (I) and an intramolecular nucleophilic catalysis of ester hydrolysis (as II) has been pointed out:[2–6]



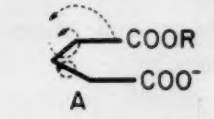
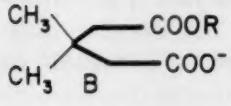
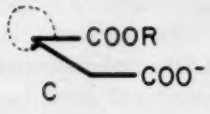
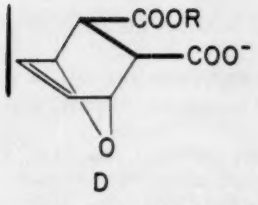
(II)

When the intramolecular model (II) is compared with its bimolecular counterpart (i.e., the catalysis of ester hydrolysis by acetate anion), it is found that II is much more efficient, due to a decrease in translational entropy in the formation of the transition state.[2,6] By analogy, the high efficiency of enzymic reactions must be due, at least in part, to a similar phenomenon, in which the ES complex brings the reacting groups together. It is commonly accepted that the enzyme must also align the reacting groups in a particularly favorable steric conformation to allow the

reaction to proceed at an optimum rate. This can be best accomplished by placing the ground state of the reacting species in approximately the same steric configuration as the transition state (steric compression). In the model II this can be accomplished by at least three different types of steric compression. The esters A, B, C, and D (Table 1) have been chosen to illustrate the kinetic effect of each.

TABLE 1

THE RELATIVE RATES OF ANHYDRIDE FORMATION (k_1) AND SOLVOLYSIS (k_2) IN THE INTRAMOLECULAR NUCLEOPHILIC CATALYSIS OF THE HYDROLYSIS OF FOUR MONO ESTERS OF DIBASIC ACIDS*, †

Ester	$\frac{k_1}{k_1 \text{ glut.}}$	$\frac{k_2}{k_2 \text{ glut.}}$
 A	1.0	1.0
 B	20	0.07
 C	230	1.46
 D	53,000	5.2

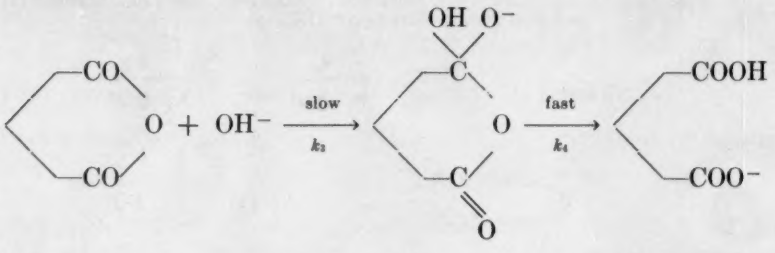
* The esters employed were the mono-*p*-bromophenyl and *p*-methoxyphenyl. Their rates of solvolysis were followed spectrophotometrically at constant pH between pH 2.6 and 7.0. Values of k_1 when plotted versus pH gave the typical sigmoid curve for a mono basic acid (carboxyl anion participation).[3-5] The values of k_1 represent the rate of hydrolysis of the mono anion. For the mono-*p*-bromo ester of glutaric acid (standard of reference for relative rates) $k_1 = 4.44 \times 10^{-3}$ min^{-1} (30°; $\mu = 0.65$ M; solvent dioxane-water 50-50, v/v).

† The values of k_2 were obtained titrimetrically at pH 6.5 and 35° in 28.5% ethanol water v/v, $\mu = 0.65$ M. For glutaric anhydride $k_2 = 0.23$ min^{-1}.

In B the β,β-dimethyl substitution hinders rotation of the COO^- and COOR groups away from each other (gem-dimethyl effect[7]) and thus sterically compresses the ground state somewhat toward the transition state. The nucleophilic displacement in C is favored over that in A because fewer bonds need be frozen into position upon entering the transition state in C as compared to A (cf. Brown, Brewster, and Schecter rule[8] dealing with relative stabilities of five versus six membered rings with exocyclic double bonds). In D, the attacking carboxyl group and the ester bond are locked together in an eclipsed conformation by the endoxo bridge. The ester

D possesses a stereochemistry very similar to that of the transition state in contrast to A which permits free rotation of the reacting groups around the three carbon-carbon bonds.

It is also noteworthy that the relative rate (k_2) for anhydride solvolysis (akin to the deacylation step in I) is not decreased with increasing compression. This may be easily explained by assuming that the attack of OH^- ion on the anhydride molecule is the rate limiting step



Thus, the compression which increases k_1 (the rate of anhydride formation) need not have an influence on anhydride opening, since the effect does not enter into the rate determining step (k_4). A completely analogous type of steric control could be operative in enzymic reactions involving double displacements.

These experimental results point to the tremendous enhancement of rate that an enzyme could achieve by fixing the reacting species in a steric conformation closely resembling that of the transition state for the reaction. A detailed account of "The Effect of Geminal Substitution, Ring Size and Rotamer Distribution on the Intramolecular Catalysis of Ester Hydrolysis" will be submitted to the *Journal of the American Chemical Society.*

* This work was supported by grants from The National Institutes of Health, The National Science Foundation, and the Upjohn Company.

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[3] Bruice, T. C., and J. M. Sturtevant, *ibid.*, **30**, 208 (1958); *J. Am. Chem. Soc.*, **81**, 2860 (1959).

[4] Bruice, T. C., *J. Am. Chem. Soc.*, **81**, 5444 (1959).

[5] Schmir, G. L., and T. C. Bruice, *ibid.*, **80**, 1173 (1958).

[6] Bender, M. L., and M. C. Neveu, *ibid.*, **80**, 5388 (1958).

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[8] Brown, H. C., *J. Org. Chem.*, **22**, 439 (1957).

*FREE RADICALS IN HEART MUSCLE MITOCHONDRIAL PARTICLES: GENERAL CHARACTERISTICS AND LOCALIZATION IN THE ELECTRON TRANSPORT SYSTEM**

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Communicated by E. U. Condon, February 3, 1960

Introduction.—The technique of electron spin resonance (ESR) spectrometry has enabled a new approach to the study of free radicals in biological and biochemical systems. There are good theoretical grounds for expecting free radicals to occur as intermediates in a number of biochemical processes, and the new technique presents an opportunity for testing these theories.

Ideally what is required is information regarding the identity of free radicals which occur in biochemical systems, and quantitative data which relate the free radical concentration to the kinetic properties of the system. Since the ESR technique is new and still in the process of development, the degree to which this ideal is approached is very largely determined by the characteristics of the available spectrometers.

In the initial discovery by Commoner, Townsend, and Pake[1] of ESR signals due to free radicals in biological materials, the spectrometer available at the time precluded the examination of samples containing more than a trace of liquid water, so that it was necessary to lyophilize the tissue samples before analysis. Measurements of this type yield useful information regarding the presence or absence of free radicals, but such static data provide only indirect evidence regarding the relationship between the free radicals and metabolic processes. In more recent studies of enzyme systems by Kubo *et al.*[2] and Bray *et al.*[3] the problem presented by the aqueous character of functional enzyme systems was solved by examining samples in the frozen state. As in the case of lyophilized samples, this approach also precludes kinetic data.

With improvements in spectrometer design it is possible to obtain ESR signals from samples containing 0.1–0.15 ml of liquid water.[4–8] The presence of liquid water, which tends to absorb the microwave radiation incident on the sample, increases the difficulty of achieving an instrument sensitivity sufficient to detect the very low concentrations of free radicals that occur in biological and biochemical systems. One way to achieve sensitivity is to scan the ESR signal relatively slowly. Ehrenberg and Ludwig[9] have reported an ESR signal in a functional aqueous system containing yellow enzyme using a scanning time of 12 min. However, such a time scale imposes a serious limitation on kinetic experiments.

With the development by Dr. Jonathan Townsend of the Department of Physics at Washington University of an ESR spectrometer of extreme sensitivity, it is now possible to detect free radicals in aqueous solutions at concentrations of 10^{-6} to 10^{-7} M, using 1.5–2 min scanning periods. Investigation of a number of functional enzyme systems by means of this spectrometer has led to evidence that free radicals occur in them, and in certain intact living cells.[5, 6, 8] However, these experiments, like the preceding ones, lack sufficient quantitative detail to permit

conclusions regarding the biochemical mechanisms which give rise to the observed free radicals.

In the present paper, and one which follows,[10] we report quantitative observations on the kinetic properties of free radicals which occur in particles derived from the sarcosomes of pig-heart muscle. The data lead to conclusions which localize the free radicals in a particular segment of the heart particle electron transport system and which describe the biochemical mechanisms in which the free radicals participate. On the basis of these conclusions, it becomes possible to deduce from the ESR data the redox potential of succinic dehydrogenase (as distinct from the redox potential of the enzyme-substrate system) and the equilibrium constant for the enzyme-substrate reaction—values hitherto unavailable from biochemical investigations of this enzyme system.

Experimental Methods.—Reagents: Cytochrome *c* was purchased from the Sigma Chemical Company and Antimycin A was obtained through the University of Wisconsin Alumni Research Foundation. Antimycin A solutions were prepared in 50 per cent to 95 per cent ethanol. All buffered solutions of salts and other reagents were prepared to contain 10^{-4} to 10^{-2} *M* Tris or phosphate buffer at a final pH of 7.4.

Heart particle preparations: Pig hearts were chilled immediately after slaughter. The ventricles were removed, cleaned of connective and adipose tissues, and ground twice. The resulting macerate was washed by stirring 200 gm. of macerate with 2 liters of water for 10 min, the supernatant liquid being removed by decantation and discarded. This washing procedure was repeated four more times. Finally the washed macerate was homogenized in a Waring blendor for 7 min with 500 ml of 0.1 *M* potassium phosphate buffer at pH 7.4. The resulting homogenate was centrifuged for 30 min at 1800 RCF $\times$ *g*. The supernatant was retained and the sediment was further homogenized in the blendor for 3 min with 300 ml of fresh buffer. This second homogenate was centrifuged as above and the first and second supernates were combined. The combined supernatants were then centrifuged at 16 to 20 $\times$ 10^3 RCF $\times$ *g* for about 1 hr. The resulting sediment, after having been resuspended in a minimal amount of 0.1 *M* potassium phosphate buffer, pH 7.4, comprises the heart particle preparation. Preparations were stored at $-20°$C until used. After grinding the heart, all subsequent operations were performed at 2–4°C.

Apparatus: The ESR spectrometer was designed by Dr. Jonathan Townsend of the Washington University Physics Department and constructed under his supervision. A brief description has been published elsewhere.[4, 8] The apparatus can detect free radicals in aqueous media at concentrations of 10^{-6} to 10^{-7} *M* or higher.

For most ESR observations, samples were contained in closed Pyrex cells which were inserted through a slot into the resonance cavity of the ESR spectrometer. These cells, which have been described earlier,[4, 8] accommodate 0.1 to 0.2 ml of sample.

In some experiments it was desirable to control the gaseous environment of the sample. This was accomplished by means of a flow system composed of a Sigma T4 peristaltic pump, glass gas exchanger, narrow bore Tygon and rubber tubing, and a U-shaped Pyrex cell which is accommodated by the slot in the resonance cavity. The sample flows through the gas exchanger into the cell, from the cell to

the receiving side of the pump, and finally is returned to the gas exchanger. The system accommodates 1 to 2 ml of liquid at variable flow rates up to 20 ml min^{-1}, depending on pump speed. ESR signals may be determined while the sample flows through the ESR cell.

The control of sample temperature during ESR studies was accomplished by the flow of gas at a desired temperature past the sample cell. Buffered solutions of potassium peroxylamine disulfonate were used as the magnetic field marker. Spectrophotometric observations were made with a Cary Model-11 recording spectrophotometer.

Results.—General properties of heart particles: The preparations of heart particles described above were found to contain 0.4 to 0.6 gm. ml^{-1} of nondialyzable material on a wet weight basis and approximately 0.07 to 0.10 gm. ml^{-1} on a dry weight basis. Upon storage the preparations exhibit succinoxidase activity and other properties relevant to the purposes of this paper for at least several days at 0°C and three weeks at −20°C.

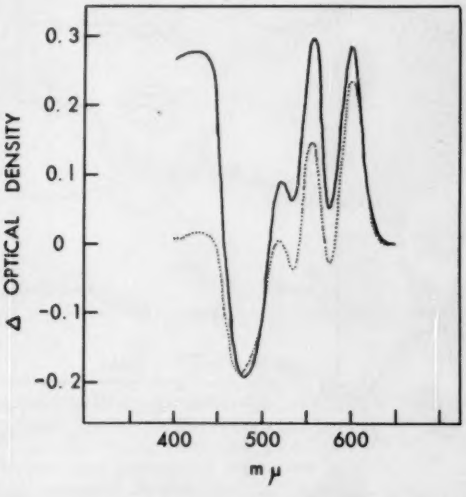

FIG. 1.—Difference spectra between reduced and oxidized heart particles. The ordinate represents the optical density of the reduced system under anaerobic conditions minus the optical density of the oxidized system under aerobic conditions. The reducing agents are sodium hyposulfite (solid curve) and 10^{-2} *M* succinate (broken curve). The heart particle preparation is diluted 3 fold with 0.1 *M* potassium phosphate buffer, pH 7.4.

Figure 1 illustrates difference spectra between an aerobic heart particle suspension and the same suspension reduced anaerobically by succinate and sodium hyposulfite. The spectra indicate that the heart particles contain the well-known components normally found in mammalian electron transport systems. The spectrum includes the 605 mμα band of cytochromes of the *a* group, the 560 mμ peak representative of the fused α bands of other cytochromes, and the strongly overlapping and mutually repressed peaks at lower wavelengths due to flavins and the γ bands of the cytochromes.

The heart particles exhibit succinoxidase and succinate-cytochrome *c* reductase activities, and therefore those components of the electron transport system which effect the transfer of electrons from succinate to cytochrome *c* and to oxygen are functional. These components include succinic dehydrogenase, cytochromes and, in the case of succinoxidase activity, cytochrome oxidase. Observation of succinoxidase activity of heart particles does not require addition of cytochrome *c*. These heart particle preparations appear to be similar to those described by Keilin and

Hartree[11] with regard to method of preparation, spectral properties, and oxidative activity.

The perceived color of dense heart particle suspensions changes from red-brown to green-brown under anaerobic conditions in the presence of succinate. After sufficient oxygenation, the reverse color change is observed, marking the exhaustion of succinate. These reversible changes in color, due mainly to the appearance and disappearance of the 605 mμ absorption band, were found to be useful indices in establishing the times required, under aerobic conditions, for the complete oxidation of succinate.

The electron spin resonance of heart particles: When a sample cell, containing about 0.05 gm. of heart particles suspended in 0.15 ml of aerated phosphate or tris

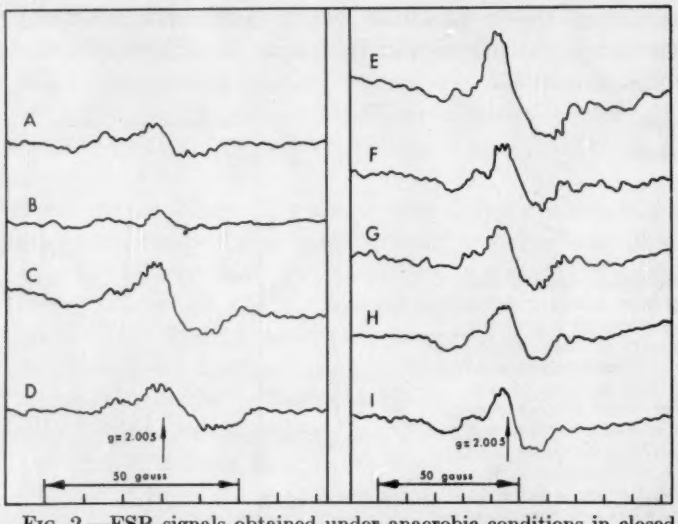

FIG. 2.—ESR signals obtained under anaerobic conditions in closed cells in the system containing heart particles and 10^{-2} *M* succinate under different instrumental conditions. The curves represent the derivative of resonance absorption with respect to magnetic field as a function of magnetic field, which increases to the right along the abscissa. The magnetic field modulation amplitude in gauss, the spectrometer's minimum response time in seconds, and the time in minutes required to scan a 100-gauss segment of the magnetic field are, respectively, the following: (*a*) 2, 1, 3.3; (*b*) 1, 1, 3.3; (*c* and *d*) 4, 1, 3.3; (*e*) 8, 0.1, 0.6; (*f*) 4, 0.1, 0.6; (*g*) 4, 0.1, 1.3; (*h*) 4, 1, 1.3; (*i*) 4, 1, 2.5. Gas phase was nitrogen. Initial oxygen concentration in aqueous phase was that of dissolved oxygen under atmospheric conditions and 25°C.

buffer, pH 7.4, is examined in the ESR spectrometer at magnetic fields in which signals due to organic free radicals are observable, no signals are detected other than the small effects attributable to the glass of the sample cell. Succinate solutions also show no ESR signal under aerobic or anaerobic conditions. However, Figure 2 shows that the addition of relatively low concentrations of succinate to aerated heart particle suspensions contained in closed cells produces readily detectable ESR signals. Due to the high succinoxidase activity of the particles, the system is anaerobic at the time ESR observations are made.

The ESR signal is centered at $g = 2.003 \pm 0.002$, and the width between regions of zero slope in the first derivative curves shown in Figure 2 corresponds to 15 ± 1.5

gauss. Figure 2 shows that the signal width and shape are not affected appreciably by variations in the amplitude of the magnetic field modulation between 1 and 8 gauss, so that the observed width can be regarded as characteristic of the signal. In the studies reported below, modulation amplitudes of the order of 8 to 11 gauss were commonly employed.

The signals shown in Figure 2 are not due to a simple Gaussian absorption. A small but reproducible asymmetry can be seen on the low field side of the main signal which suggests that more than one species of unpaired electron may be present. However, since symmetrical sub-peaks due to hyperfine splitting are not always equally resolved on both sides of the resonance center, it is possible that the signal is due to a single, complex resonance. In any case, in what follows we shall be concerned with the behavior of the main part of the signal, the magnitude of which may

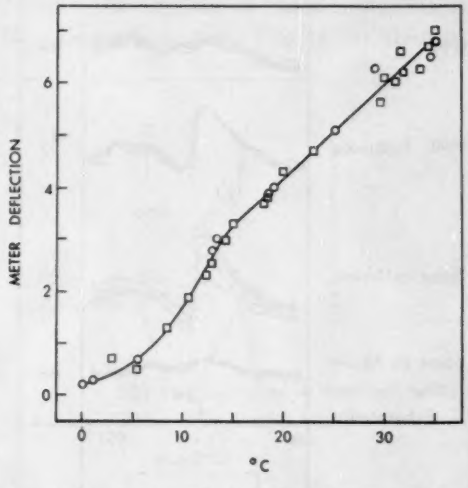

FIG. 3.—The reversible effect of temperature on the size of the ESR signals from heart particles in the presence of fumarate and succinate under anaerobic conditions in a closed cell. Fumarate/succinate mole ratio of 1; fumarate plus succinate concentration is 20×10^{-3} *M*. All points are corrected for any resonance in glass of cell. Open circles: decreasing temperature. Open squares: increasing temperature. Gas phase was nitrogen.

be estimated from the meter deflections at points of zero slope in the ESR derivative curve. This estimate provides a relative value for the concentrations of the unpaired electrons in the sample.

Comparison of the observed meter deflections with those given by standard aqueous solutions of a stable free radical indicates that heart particles contain a maximum of about 10^{-8} moles of unpaired electrons per gm. wet weight.

When heart particle suspensions are centrifuged in the presence of succinate, the ESR signal is found in the sedimented particles and not in the supernatant fluid. The signal is therefore associated with the heart particles themselves.

The *g* value and signal shape suggest that the observed ESR signal is due to organic free radicals.[12] The low concentrations of free radicals observed and the fact that the only known interaction between succinate and the heart particles involves the succinoxidase system suggest that the free radicals are associated with the catalytic activity of the heart particle and, in particular, with one or more components of the succinoxidase system. The data which follow serve to specify further the biochemical origin of the ESR signal and to establish that it results from free radicals associated with redox activity of the heart particles.

Metabolic properties of heart particle free radicals: The free radicals observed in heart muscle particles upon the addition of succinate appear to be stable in the ab-

scence of oxygen. Under this condition no diminution has been noted in the size of ESR signals from a given sample for a period of at least 7 hr at about 30 °C. Storage at 4°C for several days also appears to have no effect on the signal size as measured at about 30°C.

Figure 3 illustrates the effect of temperature on the size of the ESR signal observed under anaerobic conditions in the presence of 10^{-2} *M* succinate. Since the signal shape is not affected by temperature, the meter deflection between points of zero slope can be regarded as a measure of free radical concentration. The free radical concentration diminishes with decreasing temperature in the range from 35° to 0°C, being very nearly undetectable around 0° to 2°C. This effect is completely reversible. Contrasting with this reversible change is the irreversible effect of higher temperatures. Heating a sample to 70°C for a few minutes or to 100°C for a few seconds, which destroys the enzymatic activities of heart particles, causes

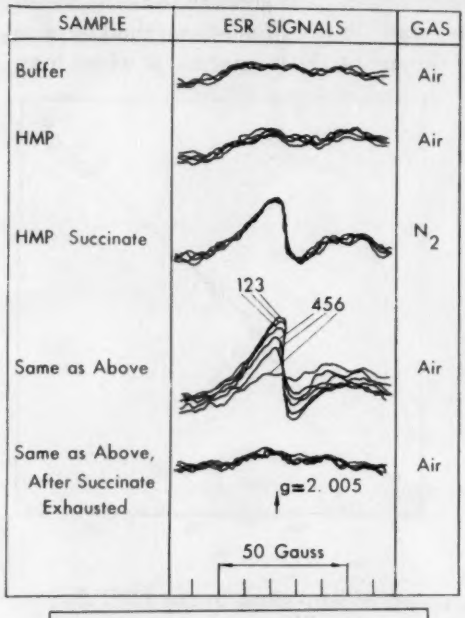

FIG. 4*a*.—ESR signals from a flowing suspension of heart muscle particles. About 2 ml. of fluid was circulated through a closed flow system consisting of a peristaltic pump, a gas exchanger, and a U-shaped ESR cell (effective volume about 0.1 ml) connected by tygon tubing. Rate of liquid pumping was about 10 ml/min. Rate of gas flow through the gas exchanger was about 100 ml/min. Uppermost figure represents tracings of 3 successive ESR runs with the system containing 0.1 *M* phosphate buffer, pH 7.4, the gas phase in the exchanger being air. The second figure shows the signals obtained when the system contains a suspension of heart muscle particles (about 0.5 gm./ml) in phosphate buffer, in equilibrium with air. The third figure represents tracings of 4 successive ESR runs after succinate ($10^{-2}M$) is added to the foregoing system, with nitrogen as the gas phase. The fourth figure represents tracings of 6 successive ESR runs, over a 20-minute period following the admission of air into the previous system. The lowermost figure shows the ESR signal observed after the succinate in the above system has been exhausted.

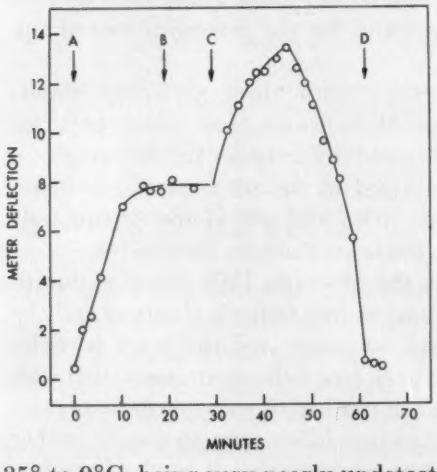

FIG. 4*b*.—Changes in the size of the ESR signal from heart particles associated with the oxidation of succinate by oxygen. Flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 15 ml min^{-1} and 100 ml min^{-1}, respectively. (a) pump and air on, 10^{-2} *M* in succinate; (b) pump off, air off; (c) pump on, air on; (d) pump off. All points are corrected for the dilution of heart particles and for any resonance in glass of cell. Aqueous phase contained initially dissolved oxygen under atmospheric conditions at 25°C.

the permanent loss of the ESR signal. These results suggest that the observed free radicals result from a temperature-dependent process associated with the redox enzymes present in heart particles.

When succinate is added to heart particles under aerobic conditions in a circulating system, results such as those illustrated in Figures 4*a* and *b* are obtained. In the presence of oxygen the signal changes in size, first increasing and then decreasing. During the time when the signal declines in size, the color of the heart particle suspension changes from green to red; this indicates the exhaustion of succinate in the preparation. The ESR signal can be restored by adding fresh succinate, the size of the regenerated signal being a function of the initial succinate concentration and of the concentration of fresh succinate. This effect is shown in Figure 5.

If oxygen is withdrawn from the system, either by stopping the circulation pump or by introducing nitrogen into the gas exchanger, the ESR signal becomes stabilized with respect to size, as shown in Figure 4*b*. Regardless of its size at the time of

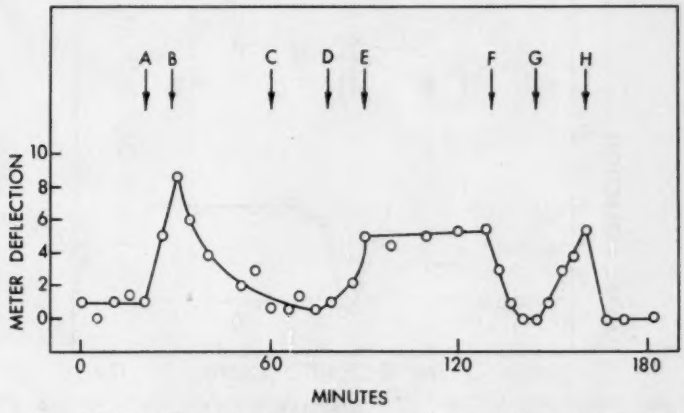

FIG. 5.—The effect of cyanide on the stability of the free radical of heart particles toward oxygen and potassium ferricyanide. Flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 12 ml min^{-1} and 150 ml min^{-1}, respectively. (Time zero to *a*) pump and air on; (*a*) 10^{-2} *M* in succinate; (*b*) air off, oxygen on; (*c*) oxygen off, 10^{-3} *M* in cyanide; (*d*) 5×10^{-3} *M* in succinate; (*e*) oxygen on; (*f*) oxygen off, nitrogen on, 15×10^{-3} *M* in ferricyanide; (*g*) 5×10^{-3} *M* in succinate; (*h*) 15×10^{-3} *M* in ferricyanide. All points are corrected for the dilution of heart particles and for any resonance in glass of cell.

anaerobiosis, the signal maintains this amplitude indefinitely thereafter. The changes in signal size upon the oxidation of succinate, therefore, are not transitory effects associated with net electron transport. The free radicals apparently arise from some enzymatic transformation involving succinate which occurs in the absence of oxygen and over-all electron transport.

The point of origin of heart particle free radicals: In order to localize the redox processes which give rise to the observed free radicals in the succinoxidase system, the effects of altering the electron transport system by means of inhibitors and artificial oxidants were determined.

Figure 5 shows the effect of cyanide on the stability of the free radicals toward oxygen and toward an alternate oxidant, potassium ferricyanide, in a circulating system. As shown in Figure 5, under aerobic conditions addition of succinate (at time

A) causes the free radical concentration to rise briefly and then fall. When cyanide and fresh succinate are added (times C and D) the ESR signal becomes stable in the presence of oxygen for an indefinite period. Thus, cyanide in the presence of oxygen has the same effect as anaerobic conditions (See Fig. 4*a* and *b*).

The addition (at time F), under nitrogen, of an amount of potassium ferricyanide in excess of the redox equivalents of succinate present in the cyanide-inhibited system results in the rapid decay of the ESR signal. However, when succinate is again added to achieve a concentration which exceeds the redox equivalents of ferricyanide present (time G), the signal again rises. Finally, the ferricyanide-induced reduction in free radical concentration is again exhibited when ferricyanide is added, in excess to the succinate, at time H.

These results show that when electron transport in the succinoxidase system is inhibited by cyanide, oxygen cannot diminish the free radical concentration. The

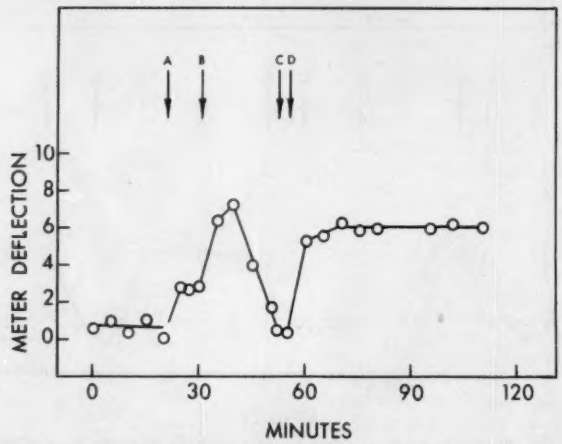

FIG. 6.—The effect of Antimycin A on the stability of the free radical of heart particles toward oxygen. Flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 12 ml min^{-1} and 100 to 150 ml min^{-1}, respectively. (Time zero to *a*) pump on, nitrogen on; (*a*) 1.25×10^{-2} *M* in succinate; (*b*) nitrogen off, oxygen on; (*c*) pump and oxygen off; (*d*) 1.25×10^{-2} *M* in succinate, 10 μg ml^{-1} in Antimycin A, pump and oxygen on. All points are corrected for dilution of heart particles and for any resonance in glass of cell. Aqueous phase initially contained dissolved oxygen under atmospheric conditions at 25°C.

events which produce the free radicals therefore lie on the succinate or reducing side of the site of cyanide inhibition, which is at the level of cytochrome oxidase.[11] This result also shows that the free radicals are not destroyed by reacting directly with oxygen.

The reduction of free radical concentration by ferricyanide in the presence of cyanide, which is counteracted by excess succinate, appears to be due to oxidative depletion of succinate. Ferricyanide can bypass the site of cyanide inhibition in the succinoxidase system and thereby oxidize succinate despite the presence of the inhibitor. The effect of ferricyanide resembles that of a redox titration and substantiates the conclusion, indicated with regard to Figure 4, that the presence of succinate is essential for the occurrence of detectable amounts of free radical.

Similar studies with Antimycin A are described in Figures 6 and 7. Antimycin inhibits both the succinoxidase and succinate-cytochrome *c* reductase activities of

heart particles at a site between succinic dehydrogenase and cytochrome *c*.[13–15]

Figure 6 shows that, like cyanide, Antimycin A (added at time D) brings about a stabilization of the succinate-induced ESR signal in the presence of oxygen. Figure 7 illustrates the effect of oxidized cytochrome *c* on the ESR signal from the heart particle system under various conditions. In these systems neither reduced or oxidized cytochrome *c* alone, nor mixtures of these, exhibits a detectable ESR signal under the spectrometer conditions employed in these experiments.

Figure 7 shows that addition of more than 12 mg of oxidized cytochrome *c* abolishes the ESR signals in the heart particle system (within 3–5 min). The same effect is observed when cyanide is present. However, in the presence of Antimycin A the ESR signal is not immediately affected by the addition of cytochrome *c*. In this case the cytochrome *c* effect does occur after a delay of 30 to 60 min. Ap-

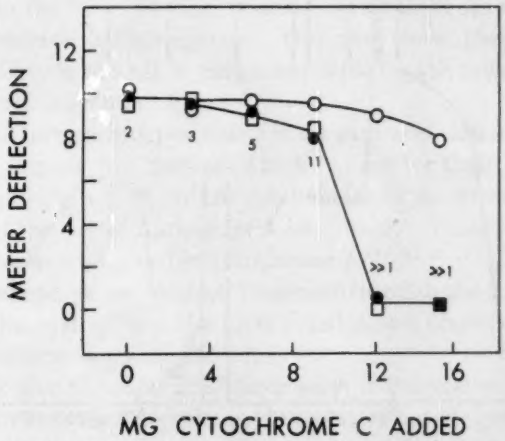

FIG. 7.—The effect of the oxidation of succinate by oxidized cytochrome *c* on the ESR signal from heart particles in the presence and absence of electron transport inhibitors. Signals are from 0.1 ml heart particle preparation in closed glass cells under nitrogen, containing 0.5×10^{-6} mole succinate and a fumarate to succinate mole ratio of 2. Solid circles: no inhibitor. Open squares: 5×10^{-4} *M* in cyanide. Open circles: 2 μg. Antimycin A per ml heart particle preparation. The latter curve reached zero meter deflection about 45 min after the addition of cytochrome *c* at the 12 to 15 mg level. Meter deflection values were recorded 2 to 10 min after the addition of solid cytochrome *c*. The numbers represent an estimate of the fumarate/succinate ratio based on a molecular weight of 13,000 for cytochrome *c*. All points are corrected for any resonance in glass of cell and represent the average of 3 to 9 values.

parently the amount of Antimycin A employed does not completely inhibit the succinate-cytochrome *c* reductase system.

The stoichiometry of the redox reactions depicted in Figure 7 substantiates further the conclusion that the disappearance of the ESR signals coincides with the complete oxidation of succinate. The systems originally contain 0.5 μ mole or 1 μ redox equivalent of succinate under anaerobic conditions. The level of cytochrome *c* required to abolish the ESR signal is about 12 mg which, on the basis of a molecular weight of 13,000 and one redox equivalent per mole,[16] corresponds also to about 1 μ redox equivalent, and is just sufficient to oxidize all of the succinate present.

The foregoing results indicate that the formation of free radicals by succinate does not require the activity of any part of the succinoxidase system which lies on the

oxidizing side of the site of Antimycin A inhibition. The only redox components known to participate in the succinoxidase system on the reducing side of the Antimycin A-sensitive point are succinic dehydrogenase and possibly a cytochrome, which may be cytochrome *b*.[17, 18]

Figure 8 illustrates the effect of malonate, a specific competitive inhibitor of succinic dehydrogenase,[19] on the succinate-induced ESR signal. When malonate is added, under anaerobic conditions, to achieve a concentration of 10^{-2} *M*, to a heart particle suspension which is 1.25×10^{-2} *M*, with respect to succinate and 0.5×10^{-2} *M* with respect to fumarate, the ESR signal is reduced about 50 per cent (time C). The effect of 1.5×10^{-2} *M* malonate is slightly greater. At these concentrations of malonate, succinoxidase activity is not completely inhibited, and when

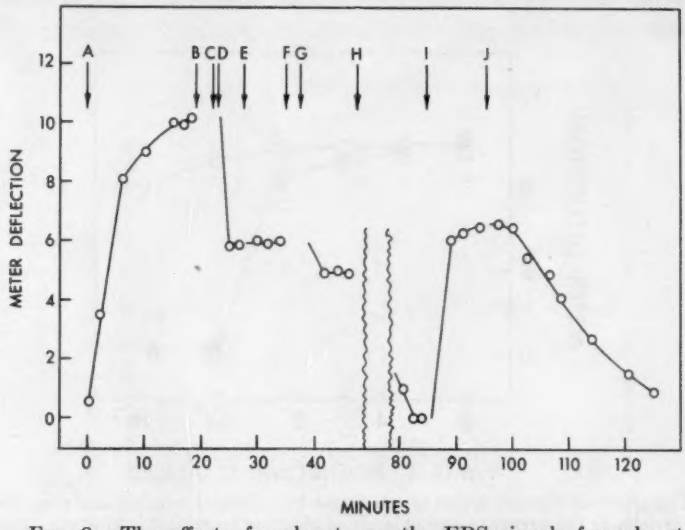

FIG. 8.—The effect of malonate on the ERS signals from heart particles. The flow system contains 2 ml of heart particle preparation. Liquid and gas flow rates are 15 ml min^{-1} and 100 to 150 ml min^{-1}, respectively. (*a*) pump and nitrogen on, 1.25×10^{-2} *M* and 0.5×10^{-2} *M* in succinate and fumarate, respectively; (*b*) pump and nitrogen off; (*c*) 10^{-2} *M* in malonate; (*d*) pump and nitrogen on; (*e*) pump off; (*f*) 1.5×10^{-2} *M* in malonate; (*g*) pump on; (*h*) nitrogen off, oxygen on; (*i*) oxygen off, nitrogen on, 0.86×10^{-2} *M* and 1.5×10^{-2} *M* in succinate and fumarate, respectively; (*j*) nitrogen off, oxygen on. All points are corrected for the dilution of heart particles and for any resonance in glass of cell. Aqueous phase initially contained dissolved oxygen under atmospheric conditions at 25°C.

oxygen is readmitted to the system (time H), the signal gradually declines to zero. When more succinate is added under nitrogen, at time I, the signal is restored, but it again disappears when oxygen is readmitted and the succinate is oxidized (time J).

Table 1 shows that the degree to which malonate reduces the free radical concentration is a function of the concurrent concentration of succinate. This competitive effect is analogous to that observed when succinic dehydrogenase activity is determined biochemically in the presence of various concentrations of malonate and succinate.

Malonate, Antimycin A, and cyanide all inhibit succinate oxidation by the electron transport system of heart particles. Of these three inhibitors, however, only

TABLE 1

THE COMPETITIVE EFFECTS OF MALONATE AND SUCCINATE CONCENTRATIONS ON THE SIZE OF THE ESR SIGNAL FROM HEART PARTICLES UNDER ANAEROBIC CONDITIONS

Malonate, $M \times 10^3$ / Succinate, $M \times 10^3$ →	Meter Deflection 2	4	6	10
0	5.2	5.9	6.2	6.6
10	2.3	3.9	4.3	4.8
20	1.4	2.4	3.2	4.3

The systems contained heart muscle preparation, succinate, and malonate as indicated, and a mole ratio of fumarate to succinate of 2. Final pH was 7.4. ESR measurements were made at 35°C in closed glass cells. Meter deflections are proportional to free radical concentration.

malonate is capable of diminishing the free radical concentration under conditions which preclude net oxidation of succinate. Cyanide and Antimycin A appear to have no direct effect on the reaction which produces the free radicals.

These observations are in keeping with the conclusion that malonate inhibits free radical formation in the heart particle systems by virtue of its well-known competitive inhibition of succinic dehydrogenase. Our data show, therefore, that succinic dehydrogenase activity is at least in part responsible for the appearance of free radicals in the heart particle system.

Discussion.—The foregoing experiments show that addition of succinate to heart particles produces organic free radicals which require for their formation the presence of succinate and the activity of the components of the succinoxidase system on the reducing side of the site of Antimycin A inhibition. These components include succinic dehydrogenase, and possibly cytochrome *b*.[13–15, 17, 18] The presence of free radicals does not depend on net electron transport through the succinoxidase system or any portion of this system, and the ESR signal is best observed under conditions which preclude net electron transport.

The free radicals give the superficial impression of considerable stability. They may be detected for extended periods as long as succinate is present, and they are not destroyed directly by oxygen. However, this stability is only apparent.

That the observed ESR signal is not due to a chemically stable free radical is shown by the reversible effects of temperature and of changes in concentrations of substrate and malonate. The effects of malonate are specific evidence that the free radicals are associated with the activity of succinic dehydrogenase.

That the observed free radicals are reactive intermediates, despite their persistence under anaerobic conditions, is evident from a consideration of the properties of succinic dehydrogenase.

This enzyme catalyzes reversible redox reactions between succinate and its oxidized product, fumarate.[20] Enzyme-catalyzed oxidation of succinate and reduction of fumarate will proceed in the absence of oxygen, so that free radical redox intermediates, if they occur, may be observed under these conditions. In the absence of oxygen there is no net oxidation of succinate and the system exists in equilibrium. Hence the parameters which govern the enzyme redox reactions will remain unchanged with time, and we may therefore expect the concentration of free radical intermediates to remain constant as well.

In the presence of oxygen, which results in net oxidation of succinate to fumarate, at least one of the relevant parameters, the fumarate/succinate ratio, varies and may in turn affect the concentration of free radical intermediates. As shown by Figure 4, when oxygen is admitted into a heart particle system containing succinate,

thereby initiating a rapid change in the fumarate/succinate ratio, the free radical concentration increases to a maximum and then declines to zero

Our results suggest, therefore, that the free radicals observed in heart particles arise from equilibrium processes catalyzed by succinic dehydrogenase and that their steady-state concentration is governed by the parameters which affect this equilibrium. The paper which follows presents quantitative data on the relation between these parameters and the free radical concentration, which confirm the foregoing conclusion and characterize the reaction mechanisms involved in free radical formation.

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*FREE RADICALS IN HEART MUSCLE MITOCHONDRIAL PARTICLES: MECHANISM OF FORMATION**

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A preceding paper[1] describes electron spin resonance (ESR) experiments on the free radicals associated with the succinoxidase system of heart particles. The results indicate that the free radicals arise from redox equilibria involving succinate and one or more components of the succinoxidase system which function on the reducing side of the site of Antimycin A inhibition. Of these components, succinic dehydrogenase, a flavin enzyme[2, 3] appears to be involved specifically in free radical formation.

In the succinoxidase system, succinic dehydrogenase mediates electron transport between the system's ultimate electron donor, succinate, and the rest of the electron transport system, possibly reacting specifically with cytochrome *b*.[4-6] In view of the ESR data described in the preceding paper, the observed free radicals might arise in either or both the coupled oxidation-reduction of the enzyme's prosthetic group with succinate and fumarate, or with a component of the electron transport system functioning on the reducing side of the site of Antimycin A inhibition.

The present paper describes experiments designed to specify the origin of the succinoxidase free radicals more precisely. In these experiments the effects of variations in the concentrations of succinate, fumarate, malonate, and heart particles on the observed free radical concentration have been determined. These data lead to conclusions which serve to characterize the reaction mechanism responsible for the appearance of free radicals in the heart particle succinoxidase system.

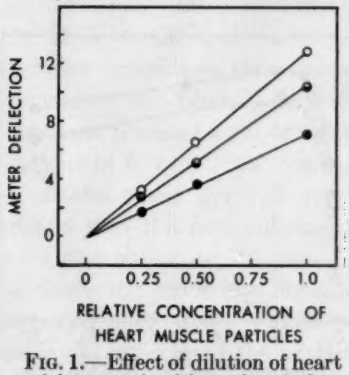


FIG. 1.—Effect of dilution of heart particles contained in a closed glass cell on the ESR signals from anaerobic systems containing fumarate and succinate. Open circles, half solid circles and solid circles represent, respectively, fumarate plus succinate concentrations of 50×10^{-3} *M*, 20×10^{-3} *M* and 2×10^{-3} *M*. The fumarate/succinate mole ratio in each case is 2. The ordinate represents the meter deflection due to the ESR signal at the magnetic field at which the deflection is at a maximum. This value is proportional to the free radical concentration. All points are corrected for any resonance in glass of cell and represent the average of 3 to 9 values.

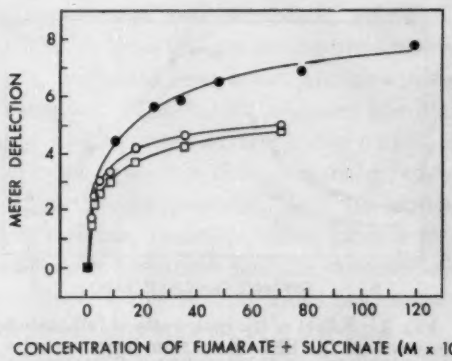


FIG. 2.—Effect of the total concentration of fumarate plus succinate on the ESR signal from heart particles under anaerobic conditions in closed glass cell. Open circles: Fumarate/succinate mole ratio of 2. Open squares: Fumarate/succinate mole ratio of 0.1. Solid circles: Fumarate/succinate mole ratio of 1 and different heart particle preparation. Ordinate as in Fig. 1. All points are corrected for the dilution of heart particles and any resonance in glass of cell and represent the average of 3 to 7 values.

Experimental Methods.—The techniques employed in these studies were the same as those described in the preceding paper.[1] Fumarase assays were made by observing changes in the optical density at 205 mμ in systems containing buffer, heart particle preparation, and 10^{-4} *M* fumarate, all at pH 7.4. Reaction and reference cuvettes were identical with the exception of the presence of fumarate in the reaction cuvette. Free radical concentration was estimated from the maximum meter deflection exhibited by the ESR signals.

Results.—Figure 1 shows the effect of the concentration of heart particles under

anaerobic conditions on the ESR signals at different total concentrations of succinate plus fumarate. The intercepts are (0, 0) and the concentrations of free radicals bears a linear relationship to the concentration of heart particles.

Figure 2 illustrates the effect of the total concentration of fumarate plus succinate on the ESR signal from heart particles under anaerobic conditions. The curves appear to have a (0, 0) intercept and their slopes everywhere decrease and approach zero slope asymptotically. These characteristics suggest a quadratic relationship between free radical concentration and the total concentration of fumarate plus succinate. The effect of the mole ratio of fumarate to succinate on the ESR signal in anaerobic systems of heart particles is illustrated in Figures 3*a* and 3*b*. It is evident that the size of the ESR signal and therefore the free radical

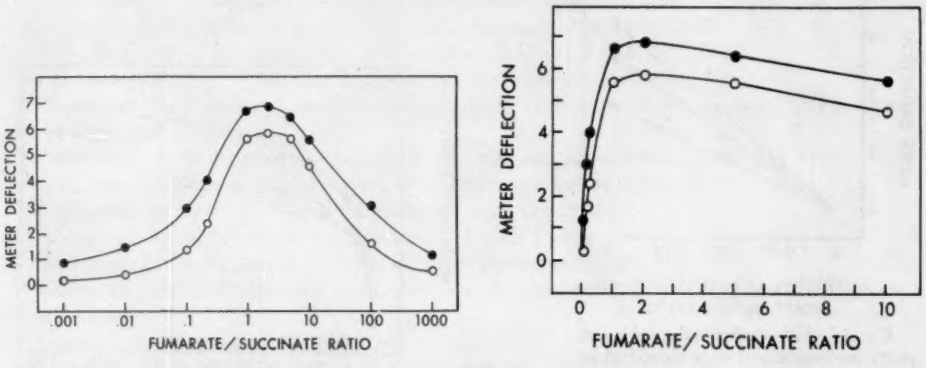


FIG. 3.—Effect of the mole ratio of fumarate to succinate under anaerobic conditions in a closed glass cell on the ESR signal from heart particles at different total concentrations of succinate and fumarate. Figure 3*a:* semilog plot. Figure 3*b:* linear coordinates. Solid circles: 47.7×10^{-3} *M* fumarate plus succinate. Open circles: 24.4×10^{-3} *M* fumarate plus succinate. Ordinate as in Fig. 1. All points are corrected for the dilution of heart particles and for any resonance in glass of cell and represent the average of 3 to 6 values.

concentration is markedly affected by the fumarate to succinate ratio. A maximum free radical concentration is observed when the fumarate succinate mole ratio is about 1.7. The position of this maximum is not affected by a 15-fold difference in total concentration of succinate and fumarate between 3×10^{-3} *M* and 5×10^{-2} *M*. On either side of this maximum, the free radical concentration declines, approaching zero when succinate or fumarate are present alone. Below the maximum the slope everywhere increases as seen in Figure 3*b*. A flex point lies above the maximum since the curve approaches zero asymptotically. The shape and limit characteristics of the curves of Figure 3*b* suggest a cubic relationship between free radical concentration and fumarate to succinate ratio.

Figure 4 illustrates the effect of malonate concentration on the ESR signals from heart particles under anaerobic conditions. The slope of the curve is everywhere negative and increases with increasing concentrations of malonate. The slope approaches zero asymptotically at zero free radical concentration. These data indicate a quadratic relationship between free radical concentration and malonate concentration.

The heart particle preparations show no detectable fumarase activity. This means that in the foregoing experiments fumarase-catalyzed destruction of fumarate does not occur, so that the concentration of fumarate remains unchanged with time.

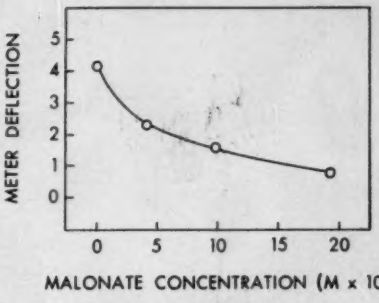
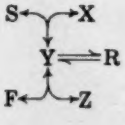


FIG. 4.—Effect of malonate on the ESR signals from heart particles in the presence of fumarate and succinate under anaerobic conditions in closed glass cell. Fumarate/succinate mole ratio of 2; 19.6×10^{-3} *M* in fumarate plus succinate. Ordinate as in Fig. 1. All points are corrected for the dilution of heart particles and any resonance in glass of cell and represent the average of 3 to 7 values.

Discussion.—Data reported in the previous paper[1] show that the magnitude of the ESR signal, and therefore the free radical concentration, observed in heart particles depends on the presence of succinate and the activity of succinic dehydrogenase and is reversibly dependent on temperatures at least between 0° and 35°C. The data presented above relate to the reversible interactions among the concentrations of free radicals, fumarate, succinate, malonate, and heart particles under conditions which preclude net electron transport. The results support the conclusion that the free radicals observed arise as the result of the redox equilibria involving succinate, fumarate, and the enzyme, succinic dehydrogenase, which catalyzes the redox equilibrium between these two compounds. Any mechanism proposed to explain the existence of the free radicals, therefore, must involve as a minimal requirement the participation of succinate, fumarate, and the oxidized and reduced forms of succinic dehydrogenase. Such a mechanism must also satisfy the various function relationships and limit conditions indicated by the data.

In what follows we shall examine several possible biochemical mechanisms and consider whether their properties conform to the requirements imposed by the data described above. Of the schemes considered, Mechanisms 1 and 2 satisfy these minimal requirements and, at the same time, are not inconsistent with what is known about the biochemistry of succinic dehydrogenase. Mechanism 2, which considers the involvement of enzyme-substrate complexes, is more probable than Mechanism 1 from a biochemical point of view.[7, 8]

Mechanism 1:



Here X, Z, S, F, Y and R represent respectively the concentrations of oxidized and reduced succinic dehydrogenase, succinate, fumarate, reaction intermediate, and free radical. The following equilibria are involved:

$$\frac{FZ}{SX} = K_1, \frac{Y}{SX} = K_2, \frac{Y}{FZ} = K_3, \text{ and } \frac{Y}{R} = K_4.$$

Let

$$F/S = Q$$

$X + Y + Z + R = T$, total succinic dehydrogenase concentration, and

$$F + S = C.$$

By solving for R in terms of the measurable variables C, T, and Q one obtains:

$$R = \frac{K_2CTQ}{K_4Q^2 + Q(K_4K_1 + K_4 + K_2K_4C + K_2C) + K_4K_1} \quad (1)$$

In this expression the free radical concentration, R, is cubic with respect to Q, quadratic with respect to C, and linear with respect to T. In these respects it conforms to the data described above. The limit and intercept properties of equation (1) also satisfy the data. A plot of R_{CT} vs Q exhibits a maximum. It can be shown that at the maximum, $Q = \sqrt{K_1}$, and therefore the position of the maximum is independent of C and T, as the data suggest. As Q approaches 0, $\left(\frac{\partial R}{\partial Q}\right)_{CT}$ approaches K_2CT/K_4K_1, that is, $\left(\frac{\partial R}{\partial Q}\right)_{CT}$ is finite and positive. As Q approaches ∞, $\left(\frac{\partial R}{\partial Q}\right)_{CT}$ approaches 0 from negative values. These properties are exhibited by the experimental curves in Figures 3*a* and 3*b*.

The properties of curves which relate R_{QT} to C are of interest in that they offer the justification for including in Mechanism 1 the equilibrium: $R \rightleftharpoons Y$, where $Y/R = K_4$. As C approaches ∞, R_{QT} approaches $T/(K_4 + 1)$. It is apparent that if Y equals zero, that is, if this equilibrium did not exist, K_4 would equal zero and the limit of R would be simply T. Physically this would mean that where the total concentration of substrate, C, is high nearly all the succinic dehydrogenase would exist as the free radical. We offer no proof that this would occur. Therefore, Mechanism 1 is offered as a more general case where this remarkable consequence need not be considered.

The effects of malonate may be treated in the following manner. It is assumed that, since malonate competes with succinate,[9] the inhibitor may interact with oxidized succinic dehydrogenase, X, but not with the reduced enzyme, Z. A fifth equilibrium is then introduced into Mechanism 1: $M + X \rightleftharpoons N$, where $N/MX = K_5$ and M and N represent, respectively, the concentrations of malonate and the complex formed between malonate and oxidized succinic dehydrogenase. Now T, total succinic dehydrogenase concentration, equals $X + Y + Z + R + N$. By solving for R with respect to Q, C, T, and M, one obtains the expression:

$$R = \frac{K_2CQT}{M(Q^2K_4K_5 + QK_4K_5) + K_4Q^2 + Q(K_4K_1 + K_4 + K_2K_4C + K_2C) + K_1K_4} \quad (2)$$

which at constant Q, C, and T, assumes the form:

$$R = \frac{a}{bM + c}$$

R is quadratic in M just as the data would seem to require. As M approaches ∞, R approaches 0. As M approaches 0, R approaches a/c, i.e. equation (2) reduces to equation (1). Similar results also are obtained if one considers that malonate competes with both succinate and fumarate[8-10] for the enzyme.

In Mechanism 1 the involvement of enzyme-substrate complexes was not specifically considered. These are taken into account in Mechanism 2.

Mechanism 2:

Here X, Z, S, F, and R have the same meaning as above. In addition, N and P are enzyme-substrate complexes, and Y_1 is a reaction intermediate with a physical significance related to that of Y in Mechanism 1. The following equilibria are sufficient for our discussion:

$$\frac{FZ}{SX} = K_1, \frac{N}{SX} = K_{10}, \frac{Y_1}{N} = K_{11}, \frac{P}{FZ} = K_{12}, \frac{Y_1}{P} = K_{13}, \text{ and } \frac{Y_1}{R} = K_{14}.$$

It is assumed that $S \gg N$ and $F \gg P$ so that $S + F = C$, as in Mechanism 1. Q has the same meaning as before, but now $T = X + Z + Y_1 + R + N + P$.

By solving R with respect to C, Q, and T, one obtains an expression which is identical with equation (1) with the following exceptions: K_2 and K_4 in equation (1) are replaced by $(K_{10}K_{11} + K_{10} + K_{10}K_{11}/K_{13})$ and $(K_{14} + K_{14}/K_{11} + K_{14}/K_{13})$, respectively. The consequences of Mechanism 2 are the same as those of Mechanism 1. The only essential difference is that the terms Y_1, N, and P are included together in the term Y of Mechanism 1 and the constants K_{10}, K_{11}, K_{13}, and K_{14} are included in K_2 and K_4 as indicated.

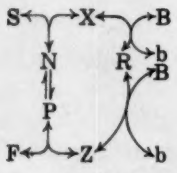
Similar results are obtained if one omits Y_1 from Mechanism 2, leaving the equilibria $N \rightleftharpoons R \rightleftharpoons P$ instead. Under this arrangement K_{11}, K_{13}, and K_{14} become zero and one must consider the expressions $N/R = K_{15}$ and $P/R = K_{16}$. Now K_2 and K_4 in Equation *1* are replaced by $(K_{10} + K_{10}K_{16}/K_{15})$ and $(K_{15} + K_{16})$, respectively.

Mechanisms 1 and 2 represent two of the simplest mechanisms which are consistent with the ESR data and with present knowledge concerning the catalytic function of succinic dehydrogenase. In both of these mechanisms the free radicals arise solely as redox intermediates in the equilibrium among succinate, fumarate, and succinic dehydrogenase, all of which are regarded as 2-electron donor-acceptors. However, other classes of mechanisms are biochemically feasible. For example, free radicals could conceivably arise from an interaction between succinic dehydrogenase and a 1-electron donor-acceptor, such as a cytochrome. Inhibitor studies described in the preceding paper show that the free radicals observed in the heart particle system are formed by reactions which lie on the reducing side of the site of Antimycin A inhibition. The only known components of the succinoxidase system which can be reduced aerobically by succinate in the presence of Antimycin A are succinic dehydrogenase and cytochrome *b*.[4, 5] There is some evidence that succinic dehydrogenase can react directly with cytochrome *b* in heart particles.[4–6, 11, 12] For these reasons we shall consider, in what follows, the possibility that the observed free radicals arise from interactions between the 2-electron donor-

acceptor (succinic dehydrogenase) and a 1-electron donor-acceptor (such as cytochrome *b*).

Consider the possibility that succinic dehydrogenase interacts with a 1-electron donor-acceptor in the manner shown in Mechanism 3.

Mechanism 3:



Here S, F, X, Z, R, N, and P have the same meaning as defined above. The terms b and B refer to the oxidized and reduced forms of the 1-electron donor-acceptor, respectively. In this case let $\beta = B + b$ and $T = X + Z + R + N + P$. It is assumed that β/T is constant in heart particles. In this mechanism, the free radical does not result directly from interactions among succinate, fumarate, and succinic dehydrogenase, but rather from interactions among this enzyme and the one electron donor-acceptor by way of bimolecular reactions. The free radical, R, here may be taken to represent the semi-reduced form of succinic dehydrogenase.

When one solves for R in terms of the experimental variables C, Q, and T, the resultant expression does not have characteristics required by the data. The most striking inadequacy involves the relationship between R_{TQ} and C. It can be shown that, while the relationship is quadratic, R_{TQ} approaches zero asymptotically as C approaches ∞. R_{TQ} is finite when C is zero. This relationship is entirely different from that depicted in Figure 2. When one considers a simpler variation of Mechanism 3 where the enzyme-substrate complexes, N and P, are neglected, the expression for R becomes independent of C. This relationship is also contradictory to the experimental results.

As indicated by the foregoing discussion, it is possible to determine whether the observed relationships between the magnitude of the ESR signal and the parameters which affect it conform with the properties of various proposed reaction mechanisms. The manner in which free radical concentration depends on substrate concentrations leads to the general conclusion that only those mechanisms in which free radicals occur solely as intermediates in an equilibrium involving succinate, fumarate, and succinic dehydrogenase, conform with the observed results. Hence, in the heart particle system, we can exclude specifically as a possible source of the observed free radicals, the equilibrium redox interactions of succinic dehydrogenase with (*a*) 1-electron donor-acceptors, such as cytochromes, and (*b*) any 2-electron agents except, of course, the substrates, fumarate, and succinate.

If the above conclusions are correct it should be possible to calculate from the ESR data the over-all equilibrium constant, K_1, for the reaction: succinate + oxidized succinic dehydrogenase $\rightleftharpoons$ fumarate + reduced succinic dehydrogenase. From the equilibrium equations which describe Mechanisms 1 and 2, it can be shown that $Q^2_{R\ \max} = K_1$, where $Q_{R\ \max}$ is the fumarate/succinate ratio which yields a maximum free radical concentration (or ESR signal). The value (1.72) of $Q_{R\ \max}$ ascertained from the data of Figure 3*a* yields a value of 2.95 for K_1. From this value one can calculate the free energy, ΔF°, and the redox potential, $\Delta E'_0$,

for the reaction. This leads to a value of -670 cal. mole^{-1} for $\Delta F°$, and $+.015$ volts for $\Delta E'_0$ at pH 7.4 and 35°C.

$\Delta E'_0$ is defined as the difference between the E'_0 for the enzyme redox couple (reduced enzyme $\rightleftharpoons$ oxidized enzyme $+ 2H^+ + 2e$) and the E'_0 for the substrate couple (succinate $\rightleftharpoons$ fumarate $+ 2H^+ + 2e$). Since E'_0 for the succinate-fumarate couple, which is known from potentiometric data, is $0.00v$ at pH 7,[13] E'_0 for the enzyme couple is $+.015v$ at this pH. It will be noted that this value lies between the E'_0 for the fumarate-succinate couple ($0.00v$) and the E'_0 for the cytochromes which occur in the succinoxidase system ($+0.1$ to $0.3v$).[14-18] This relationship is to be expected from the known sequence of electron transport in this system: substrate-enzyme-cytochromes. That the value of E'_0 for the enzyme, independently determined from ESR data, conforms with this expectation, tends to confirm our conclusions regarding the reaction mechanism responsible for the observed free radicals.

These considerations lead to the following conclusions regarding the free radicals observed in heart particles in the presence of succinate: (1) The free radicals arise in the equilibrium among succinate, fumarate, and succinic dehydrogenase. (2) The free radicals constitute one or more particular electron configurations of a complex composed of succinic dehydrogenase and its substrate. (3) The free radicals do not arise from the reaction between succinic dehydrogenase and redox systems which, in the succinoxidase system, are capable of reoxidizing the reduced enzyme. Specifically the data rule out the origin of free radicals in the reaction between succinic dehydrogenase and either 1-electron systems such as cytochrome *b* or 2-electron systems such as quinones or another flavin.

Although our data do not further specify the electron configuration of the dehydrogenase-substrate complex which is responsible for the observed ESR signal, certain possible arrangements merit discussion.

In the formation of the free radical enzyme-substrate complex, two molecules, in which all electrons occur in pairs, combine to form a structure which gives rise to an ESR signal and must therefore contain one or more unpaired electrons. One possible mechanism is the formation, within the complex, of a biradical containing two unpaired electrons. The ESR signal yielded by such a structure will depend on the location of the unpaired electrons within the complex. Generally, unpaired electrons separated by about 4A° or less will interact sufficiently to yield an ESR signal so broad as to escape detection in the spectrometer.[19]

When the separation is sufficient to minimize interaction, the resultant signal may be of two types. If the two unpaired electrons occur in similar atomic environments, at least with respect to those nuclei capable of magnetic interaction with the electron (e.g., *H*, *N*), they may yield identical ESR signals, which superimpose to form the observed signal. If the atomic environments of the two electrons are distinctive, two separable signals, of approximately equal intensities, should result. The ESR signal observed in the heart particles shows no evidence that it is comprised of two equal signals.[1] A possible explanation of the observed results is, therefore, that the enzyme-substrate complex contains two unpaired electrons which are in equivalent atomic environments, and which do not interact appreciably with each other.

An alternative explanation takes into account the possible mediation of electron

transfer by the iron atoms which occur in succinic dehydrogenase.[8] The enzyme molecule contains 4 iron atoms, two of which are firmly bound.[8, 10] Singer and coworkers [10, 20] have suggested a reaction mechanism in which it is supposed that the iron atoms may mediate electron exchange among succinate, fumarate, and the flavin prosthetic group within the enzyme-substrate complex of succinic dehydrogenase. In this case the following equilibrium may occur: (Succinate-Flavin-Fe^{+++}-Fe^{+++}) $\rightleftharpoons$ (Fumarate-Flavin-Fe^{+++}-Fe^{++})* $\rightleftharpoons$ (Fumarate-Flavin H_2-Fe^{+++}-Fe^{+++}) $\rightleftharpoons$ (Fumarate-Flavin-Fe^{++}-Fe^{++}). (In these and subsequent configurations, the asterisk denotes an unpaired electron.) In this scheme an intermediate form of the enzyme-substrate complex, (Fumarate-Flavin-Fe^{++}-Fe^{+++})*, contains one unpaired electron and will exhibit an ESR signal typical of an organic free radical. This proposal is also consistent with our experimental observations.

It is significant that the ESR data rule out the occurrence of detectable concentrations of free radicals in reactions involving succinic dehydrogenase and a 1-electron donor-acceptor. A possible reaction of this type is the redox interaction between succinic dehydrogenase and cytochrome *b*.[4-6] One of the chief arguments introduced by Michaelis[21] in his original proposals that free radicals must occur as redox intermediates is that they provide a reasonable mechanism for coupling a 2-electron system (such as succinic dehydrogenase) to a 1-electron system (such as a cytochrome). It is of interest, therefore, to consider possible explanations for the absence of a detectable free radical in this reaction.

Here too, the proposal of Singer *et al.*[10, 20] regarding the possible participation of iron atoms in succinic dehydrogenase activity is pertinent. These workers propose,[10] on the basis of inhibitor and oxidant specificity studies,[8, 20] that ferrous iron atoms exist in the reduced enzyme so that the prosthetic group may be represented as (Flavin-Fe^{++}-Fe^{++}). It is believed that the existence of ferrous atoms in the prosthetic group enables the reduced enzyme to interact efficiently with 1-electron oxidants. The oxidation of the reduced enzyme by 1-electron oxidants may be supposed to occur in two successive 1-electron-1-electron reactions to form (Flavin-Fe^{++}-Fe^{+++}) as intermediate and (Flavin-Fe^{+++}-Fe^{+++}), which represents oxidized succinic dehydrogenase, as the final product. Neither the intermediate nor the final product is a free radical, as required by our ESR data. Our data indicate that possible free radical intermediates of the succinic dehydrogenase prosthetic group, such as (Flavin)* or (Flavin-Fe^{+++}-Fe^{+++})*, do not exist in detectable concentrations in the heart particle system. (See Mechanism 3.)

It is worth noting that apart from evidence regarding the participation of free radicals in enzyme reactions, ESR data are also capable of yielding reaction parameters not easily attainable by other means. The peculiar spectral properties of succinic dehydrogenase[20] do not permit an accurate determination of the ratio of oxidized to reduced enzyme in the presence of succinate and fumarate. In the absence of this information, neither the equilibrium constant for the reaction between the enzyme and its substrate, nor the redox potential of the enzyme itself can be calculated. As shown above, the ESR data lead to reasonable estimations of these values, even though the enzyme is present as part of the complex electron transport system, and no spectrophotometric data on its oxidation state can be obtained.

The foregoing results suggest certain generalizations regarding the circumstances which govern the occurrence of detectable concentrations of free radicals in oxidation-reduction enzyme systems. In this respect the most relevant outcome of the above experiments is that the ESR signal associated with the succinic dehydrogenase enzyme system in heart particles is due to a free radical form of an enzyme-substrate complex. This conclusion leads to the generalization that factors which affect the concentration of this complex will govern the intensity of the ESR signal.

This generalization may be employed to account for most of the results of the ESR experiments on redox enzyme systems that have been reported thus far which include the necessary kinetic observations.

For an enzyme system in an equilibrium condition, the following relationships hold: oxidized enzyme + reduced substrate $\rightleftharpoons$ enzyme-substrate complex $\rightleftharpoons$ reduced enzyme + oxidized substrate. Accordingly, the concentration of the enzyme-substrate complex is a function of the product: [oxidized enzyme][reduced substrate] or of the product: [reduced enzyme][oxidized substrate]. As pointed out in the earlier discussion of Mechanisms 1 and 2, the concentration of the enzyme-substrate complex will go through a maximum at a particular reduced substrate/oxidized substrate (or oxidized enzyme/reduced enzyme) ratio depending, among other things, on the difference between the redox potentials of the enzyme and substrate redox couples. Where the difference is small, the ratio at which the maximal concentration of the complex occurs will be close to 1. If the difference in redox potentials is large, the required ratio becomes considerably smaller or larger than 1. According to the reaction described in Mechanisms 1 and 2, the free radical form of the enzyme-substrate complex is in equilibrium with the ordinary form of the complex. The free radical concentration is therefore proportional to the concentration of the ordinary enzyme-substrate complex, the proportionality constant being, presumably, characteristic for a given enzyme system.

Hence, for an enzyme system in equilibrium conditions, the concentration of free radical intermediate, and therefore the intensity of the observed ESR signal will depend on the variable factors which govern the concentration of the enzyme-substrate complex: (1) the absolute concentrations of enzyme and substrate and (2) the oxidized enzyme/reduced enzyme ratio. The free radical concentration will also depend on two invariant factors, which are characteristics for a given enzyme: (3) the affinity of the enzyme for its substrate and (4) the proportionality constant for the equilibrium between the ordinary form of the enzyme-substrate complex, and its free radical form.

Only when the ratios between oxidized and reduced enzyme and substrates in an equilibrium system are such as to yield a maximal concentration of the free radical-substrate-enzyme complex will a relatively intense ESR signal be observed. Since these ratios remain constant in equilibrium conditions, the signal will persist at a constant intensity as long as the enzyme remains unaffected by the experimental conditions. This situation is typified by the succinic dehydrogenase of the heart particle system in conditions of zero electron transport.[1]

Two types of situations may occur in enzyme systems that are in non-equilibrium conditions and undergo net oxidation or reduction. The distinction depends on how close to equilibrium the system maintains itself as the reaction progresses. If the system is such that the reaction progresses with the system nearly in equi-

librium, the free radical concentration will be governed by the same parameters which are controlling in the case of true equilibrium, i.e., the reactant concentrations, and the oxidized enzyme/reduced enzyme ratio. An experimental test for this state is that the free radical concentration is not altered significantly when equilibrium conditions are imposed on a non-equilibrium system. An example of this case is the heart particle system under aerobic conditions. When oxygen is excluded so that equilibrium conditions are established, no significant change in the size of the ESR signal is observed. (See Fig. 4 in the preceding paper[1].)

In a non-equilibrium enzyme system in which there is a considerable difference between the rates of the forward and reverse reactions, the concentration of the enzyme-substrate complex, and of its free radical form, will depend on factors which govern the rate of the predominant reaction. In general the concentration of enzyme-substrate complex will show a linear dependence on enzyme concentration and a saturation with increasing concentration of the substrate for the predominant reaction. A test for this state is the observation that the free radical concentration varies significantly when conditions leading to equilibrium are imposed on a non-equilibrium system. An example is the earlier investigation of lactic oxidative decarboxylase[22, 23] which showed that the size of the ESR signal is proportional to the enzyme concentration, and increases to a maximum with increasing lactic acid concentration. In this case the ESR signal is proportional to the net rate of oxidation.

On the basis of these criteria it is probable that in earlier ESR experiments with the cytochrome reductase system[24] and the old yellow enzyme system,[25] essentially equilibrium conditions were controlling while non-equilibrium conditions prevailed in the case of the alcohol dehydrogenase system.[24]

These considerations show that an ESR signal due to a free radical form of the complex between a redox enzyme and its substrate may exhibit a wide range of relationships to the net oxidative activity of the system, depending on the specific thermodynamic conditions which govern the process. A corresponding variety may be expected among the situations encountered in ESR investigations of intact living cells.

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THE SUSTAINED GROWTH OF HUMAN AND ANIMAL CELLS IN A PROTEIN-FREE ENVIRONMENT

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Although a few strains of mammalian cells have been grown in a protein-free medium,[1–7] most of the cultures now available require protein, usually added as whole or dialyzed serum. The role of that protein is not clear. It has been found to promote the adhesion of cells to a glass surface, and their subsequent flattening;[8, 9] but this is not its only or perhaps even primary function, for it is also required by cells growing in suspension. Since the protein amino acids are utilized to only a minor degree for the synthesis of cell protein,[10] it seemed possible that it might be providing one or more essential compounds of small molecular weight, either initially bound to the protein, or formed by its degradation. This was borne out by the present experiments, which show that protein as such is not required for the growth of the human or animal cells so far studied. Its primary function is to provide as yet unidentified compound(s), sufficiently small to pass through a cellophane membrane, and the provision of which is promoted by the addition of a pancreatic extract.

Methods.—The dual culture apparatus used in most of these experiments was a modification of that used by Nurmikko[11] and by McLimans *et al.*,[12] and consisted of two cylindrical culture vessels, each with a horizontal side tubulation terminating in a ground glass flange. Sterile silicone grease was spread on each flange, a cellophane membrane made from $^{24}/_{32}''$ Visking clear cellophane tubing was interposed, and the two vessels were then joined. The cell culture in each cylinder was kept dispersed by a suspended and free-spinning magnetic bar. The courtesy of Dr. William F. McLimans in providing a prototype model in advance of publication is greatly appreciated. The cell lines used were human strains KB and HeLa, a cloned subculture of HeLa (S3), and a mouse fibroblast, strain L 929. The suspension cultures used as inoculum were grown in a minimal basal medium containing the 28 demonstrably essential growth factors[13] supplemented with 5 per cent dialyzed human or horse serum.

In setting up the experiments, a culture in the logarithmic phase of growth was centrifuged, and the cells re-suspended in 100 ml of the protein-free basal medium to a count of 200,000 cells/ml. This was then placed in one arm of the culture vessel. The other arm contained 100 ml of uninoculated medium supplemented with 1–5 per cent dialyzed serum or a mixture of dialyzed serum and pancreatic extract. The latter was added as "Viokase," a powdered pancreatic extract prepared by the Viobin Corporation (Monticello, Illinois), used at a final concentration of 0.1–0.25 per cent powder. The insoluble portion was removed from the stock "2.5%" solution in 0.85 per cent NaCl by centrifugation prior to dialysis and sterilization through a sintered glass filter.

The cultures were counted daily, and replaced with enough fresh medium to keep the counts in the range 180,000–250,000 cells/ml. The fluid in the "feeder" compartment was replaced to an equal degree, but with a minimum 20 per cent replacement even when the cell culture required no dilution.

TABLE 1

THE GROWTH OF CELLS IN A PROTEIN-FREE MEDIUM EQUILIBRATING ACROSS A CELLOPHANE MEMBRANE WITH A "FEEDER" COMPARTMENT CONTAINING SERUM PROTEIN

	Composition of "Feeder" Compartment			
Dial. serum (5–20%)	+	+	+	+
"Non-essential" amino acids*	0	+	0	+
Pancreatic extract* (0.25%)	0	0	+	+
Cell line studied ↓	**Growth in Protein-free Compartment**			
HeLa	0	0	+	+
HeLa-S3	0,0	0,0,0	+	+
929	0,0,0	0,0,0,0,0	+,+	+
KB	0,0,0,0,+	+,+	+	+,+

* Alanine, aspartic, asparagine, glutamic, glycine, proline, and serine, each at 0.1 mM.
† "Viokase," made by Viobin Corporation, Monticello, Illinois. A large part of the powder fails to dissolve, and was removed by centrifugation prior to dialysis and glass filter sterilization.

Results.—1. *The growth of human and animal cells in a protein-free environment, separated by a cellophane membrane from a "feeder" compartment containing serum protein:* When cells were grown in suspension in a basal medium containing only the 28 demonstrably essential and chemically defined factors (amino acids, vitamins, salts, and glucose),[13] separated by a cellophane membrane from the same medium supplemented with serum protein, growth was occasionally obtained with one of the 4 cell lines tested (cf. first 2 columns in Table 1, and middle section of Fig. 1). In the successful cultures, the essential growth factor(s) deriving from the protein, and diffusing through the cellophane membrane, were apparently being provided in growth-limiting concentration. Thus, in one experiment, the generation time decreased progressively from 3.6 to 2.7 to 2.0 days as the concentration of dialyzed serum in the "feeder" compartment was increased from 5 to 10 to 20 per cent. Although the addition of the 7 nutritionally non-essential amino acids (alanine, aspartic acid, asparagine, glutamic acid, glycine, proline, and serine) each at 0.1 mM, often promoted growth, this was neither regular nor reproducible (Table 1). Preliminary equilibration of the cell-free fluids in the 2 compartments for 24 hr at 37° prior to inoculation of the protein-free compartment also had no regular effect.

In the meantime, it had been found[14] that when sterile dialyzed serum was stored

at icebox temperatures, free amino acids gradually appeared, most of which reached concentrations of 0.03–0.08 mM after 6 weeks. The rate of that proteolysis was materially increased at 37°. These results suggested that the growth-promoting effect of serum in the foregoing experiments might have been due to a similar slow breakdown of protein, and might be materially increased by adding appropriate enzymes to the serum-containing "feeder" compartment. The latter proved to be the case.

2. *The growth-promoting effect of pancreatic extract added to the "feeder" serum-containing compartment:* All four cell lines tested could regularly be grown in a protein-free medium, apparently indefinitely, if the culture was equilibrated across a dialysis membrane with the same medium supplemented with as little as 1 per cent dialyzed serum and 0.1–0.25 per cent of a pancreatic extract ("Viokase"). The growth-promoting activity of the enzyme preparation was not affected by its preliminary dialysis. Illustrative experiments with the HeLa-S3, KB, and L-929 strains are summarized in Figure 1. In other experiments, cells have been cultured for periods of up to 48 days, during which time they increased 15 millionfold.

TABLE 2

THE EFFECT OF THE DIALYZED SERUM CONCENTRATION IN THE "FEEDER" COMPARTMENT ON THE GENERATION TIME OF CELLS IN THE PROTEIN-FREE COMPARTMENT

	Pancreatic Extract* in "Feeder" Compartment	"Non-essential" Amino Acids	Concentration of Dialyzed Serum in "Feeder" Compartment: 20	10	5	2	1.5	1	0.8
			Generation Time, Days						
HeLa-S3	+	+	...	...	1.5	1.4	1.6	1.7	...
929	+	0	...	2.1	1.9	2.5	...	...	...
	0	+	...	...	1.9	...	...	...	...
KB	0	0	2.0	2.7	3.6	...	...	...	...
	+	+	2.6	...	4.2	...	...	...	...
	+	0	...	...	1.9	...	2.9	3.7	∞
	+	+	...	...	2.3	1.8	1.3	1.7	∞

The same minimal and chemically defined growth medium,[13] containing the 28 essential growth factors, was present in both compartments.
* Dialyzed Viokase at 0.25%.

When the enzyme preparation was removed from the "feeder" compartment, the growth rate slowed within a few days and the cells usually died.

The generation time of cells growing in the protein-free compartment, expressed as a function of the serum concentration in the "feeder" compartment, is shown in Table 2. As there shown, the provision of 1 per cent dialyzed serum (approximately 0.07 per cent protein) in the "feeder" compartment, supplemented with 0.1 per cent pancreatic extract, sufficed for sustained and rapid growth. Lower concentrations of serum were ineffective.

Discussion.—It was clear from the rapid proliferation of the cells in the protein-free compartment that this growth was not due to the selective multiplication of a small fraction of the population, but involved most of the original inoculum. Further, when cells which had been growing in the protein-free environment for 4 weeks were then placed in ordinary monolayer or suspension cultures, they died in the absence of added serum protein, and grew only at the normally effective concentrations.

As indicated by the growth-promoting and usually essential role of the pancreatic enzymes, the essential growth factor(s) which were diffusing into the cell culture

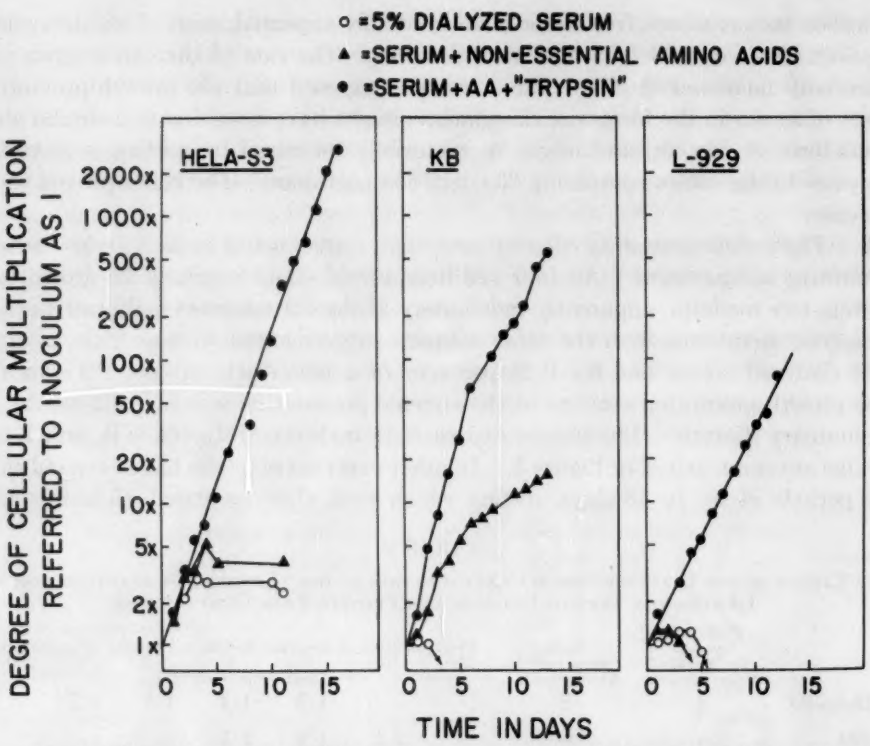

FIG. 1.—The rapid and sustained growth of mammalian cells in a protein-free medium, continuously equilibrated across a cellophane membrane with the same medium supplemented with serum protein and a pancreatic extract. The basal serum-free medium is one arm of the dialysis apparatus was equilibrated for 24 hr at 37° against either (*a*) the same growth medium supplemented with 5 per cent dialyzed horse serum along, (*b*) the dialyzed serum medium plus the 7 nutritionally nonessential amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine) each at 0.1 mM, and (*c*) the dialyzed amino acid serum medium plus "trypsin" (0.25% Viokase). After 24 hr, identical serum-free inocula, prepared by the centrifugation of suspension cultures in the logarithmic phase of growth, were added to the 3 serum-free culture arms. Thereafter, the serum-free culture fluid was removed and replaced as necessary at 1- to 2-day intervals to keep the cell count in the range 180,000–250,000 per ml. Simultaneously, the fluid in the "feeder" compartment was replaced, and to the same degree, with the appropriate serum-containing medium.

from the serum- and enzyme-containing "feeder" compartment were clearly not unchanged proteins. When cell-free medium was equilibrated for 24 hr across a cellophane membrane with medium supplemented with 20 per cent serum, with or without added pancreatic extract, the protein-free compartment still failed to give a visible turbidity either on heating, or on the addition of trichloracetic acid or sulfosalicylic acid. Further, complement fixation tests on the fluids on the two compartments, using rabbit antisera versus horse or human serum, showed no antigenically active protein on the one side of the membrane, while the fluid in the serum-containing compartment gave a positive reaction at dilutions of 1:1000–1:8000.

There was thus no evidence for the diffusion across the cellophane membrane of compounds behaving chemically or immunologically like serum protein. The essential growth factor(s) dialyzing across the membrane may have been originally bound to the serum protein, or may have been formed from it on incubation. The

growth-promoting activity of streptogenin for certain strains of lactobacilli and streptococci illustrates the latter possibility; while there are at least two analogies for the possibility that preformed growth-active substances were initially bound to the serum protein. Saprophytic treponemeta can be grown in a chemically-defined medium supplemented only with serum albumen; and, in that case, the albumen has been shown to serve solely as the carrier for an essential but toxic lipid.[15] More recently, it has been found that serum protein binds half-cystine residues which permit the growth of human and animal cells in an otherwise cystine-free medium.[16]

Whether the compounds necessary for cell growth were initially bound to protein, or were formed by its degradation, their provision to the cells was greatly accelerated by the addition of a dialyzed pancreatic extract to the "feeder" compartment, presumably because of the resulting proteolysis. Since dialyzed serum contains proteolytic enzymes,[14] it is possible that a similar degradation of serum protein may be directly responsible for its growth-promoting effect in ordinary cell cultures.

The regularity with which the suspension cultures so far tested can be grown in a protein-free environment suggests that, if intact protein is indeed necessary for cell growth in a monolayer, its primary role may be to permit the initial adhesion and flattening of the cells[8, 9] rather than their subsequent growth.

Studies are continuing with respect to the growth-promoting activity of proteins other than those in serum, the specific enzyme(s) in the pancreatic extract which are active, and the chemical nature of the postulated growth-active substance(s) formed. A number of crystalline proteolytic enzymes have had qualitatively the same effects as the crude pancreatic extract; and a number of serum protein fractions have been as effective as whole serum.

Summary.—1. Human (HeLa, HeLa-S3, KB) and mouse (L-929) cells could be regularly grown in suspension culture in a protein-free and chemically defined basal growth medium, if that culture was equilibrated across a cellophane membrane with medium containing 1–5 per cent dialyzed serum and a dialyzed pancreatic extract ("Viokase"). Only occasional and relatively slow growth was obtained when the enzyme preparation was omitted from the "feeder" compartment containing protein.

2. It is suggested that the primary role of serum protein in suspension cultures of mammalian cells is to provide essential growth factor(s) of small molecular weight, either initially bound to the serum protein, or formed from it on proteolysis.

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*METABOLISM AND MORPHOLOGY OF RIBONUCLEOPROTEIN PARTICLES FROM THE CELL NUCLEUS OF LYMPHOCYTES**

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Incubation of isolated nuclei of calf thymus lymphocytes with C^{14}-labelled precursor compounds results in rapid labelling of the proteins and ribonucleic acids (RNA) of the nuclei.[1] Some of the proteins and RNA can then be extracted from the nuclei by neutral buffer solutions.[1] This paper describes the differential ultracentrifugation of such extracts which results in the isolation of classes of ribonucleoprotein particles, each of which can be characterized by its ease of extraction, its sedimentability, its composition of protein and RNA, and its rate of intranuclear protein and RNA metabolism. Unlike cytoplasmic ribonucleoprotein particles which are potassium-dependent,[2] these nuclear particles require a sodium environment for their metabolic activity in the nucleus; furthermore, unlike cytoplasmic particles,[3, 4] the intranuclear metabolism of these nuclear particles is resistant to preincubation of the nuclei with ribonuclease (RNAase) but sensitive to pre-incubation of the nuclei with deoxyribonuclease (DNAase).

It is also shown here that after extraction from the cell nucleus, the isolated nuclear ribonucleoprotein particles remain capable of active protein and RNA metabolism in a nucleus-free system.

Previous studies of the cell nucleus by electron microscopy have demonstrated the occurrence of discrete particles of 100–300 Å diameter in the nucleolus,[5–7] on the lateral loops of lampbrush chromosomes,[8] on the chromosomal rings of Balbiani,[9] in the blebs[10] and pore annuli[11, 12] of the nuclear membrane, and throughout the nuclear sap.[13] Particles of similar size are shown here to be extracted from the nuclei of calf thymus lymphocytes, and to be composed of sub-units of 15–25 Å diameter.

The heterogeneity of the RNA of the cell nucleus has been shown previously by radioautography of labelled nuclei,[14] by isolation of labelled nuclear RNA species,[15, 16] by determination of the base composition of isolated nuclear RNA species,[17] and by histochemical staining of nuclear ribonucleoprotein species throughout the mitotic cycle.[18] Composition and metabolic heterogeneity is also

found here in isolated nuclear ribonucleoprotein particles and presumably reflects distinct origins and functions for the different particles.

Materials and Methods.—Isolation and incubation of nuclei: All procedures other than incubation were performed at 2°C. Nuclei from calf thymus lymphocytes were isolated in 0.25 *M* sucrose–0.003 *M* $CaCl_2$[1] and were incubated at 37°C in the presence of such C^{14}-labelled precursors as DL-alanine-1-C^{14}, DL-lysine-1-C^{14}, DL-leucine-1-C^{14}, orotic acid-6-C^{14}, or adenosine-8-C^{14} in the buffered sucrose-glucose medium supplemented with sodium[1] as described in detail in the legends to the figures.

Purification and extraction of nuclei: The uptake of C^{14}-precursors was stopped by rapid chilling, the incubation medium containing cytoplasmic fragments removed by centrifugation, the nuclei washed once with cold incubation medium, and the nuclei then successively extracted three times by blending 1 min at 7,500 rpm with 5 volumes of cold 0.1 *M* Tris (hydroxymethyl) aminomethane chloride (Tris Cl) buffer, pH 7.1. The resulting three Tris extracts were each separated from the nuclei by centrifugation at 1,000 *g* for 10 min. The extracts were then each purified by centrifugation at 3,000 *g* for 30 min to remove contaminating whole nuclei and particles greater than 7000 S.

Differential ultracentrifugation of extracts: Each of the three Tris extracts was subjected to successive differential ultracentrifugation in the Spinco Model L Ultracentrifuge, No. 40 head, at 2°C. Pellets were collected after successive centrifugations at 10,000 g_{av}, 33,000 g_{av}, and 105,000 g_{av} for 30 min, and at 105,000 g_{av} for 5 hr and 16 hr. The pellets formed were then washed twice with cold 0.1 *M* Tris (Cl), pH 7.1. Ranges of sedimentation constants for the particles in the pellets were estimated from the performance characteristics of the Spinco Model L No. 40 Rotor.

Measurement of protein, RNA, DNA, and radioactivity: Cold trichloroacetic acid was added to the pellets and supernates to final concentration of 5 per cent trichloroacetic acid (TCA). Radioactivity in protein or RNA was measured as previously described[15] in a thin-window gas-flow counter, and the counts were corrected for self-absorption.[19] Deoxyribonucleic acid (DNA) was determined by the diphenylamine reaction,[20] and RNA by the *p*-bromphenylhydrazine reaction[21] on TCA hydrolysates obtained after heating for 30 min at 90°C. Protein was determined as previously described.[1]

Electron microscopy: Electron micrographs of the ultracentrifuge pellets were obtained after fixation of the pellets in Veronal-buffered osmium tetroxide pH 7.3, dehydration in alcohol, embedding in *n*-butylmethyl methacrylate, and sectioning on a Porter-Blum microtome. A Phillips Model EM-100 A electron microscope was used with intermediate lens and Kodak D-426 film yielding microscopic magnifications to 20,000 X. Subsequent magnifications to 240,000 X were obtained photographically.

Studies on a nucleus-free system: For studies of leucine-1-C^{14} incorporation into proteins of isolated nuclear ribonucleoprotein particles in a nucleus-free system, the nuclei were isolated in 0.25 *M* sucrose–0.003 *M* $MgCl_2$, and freed of contaminating cytoplasmic fragments by two washings in an incubation medium of the following composition: 0.1875 *M* sucrose, 0.02 *M* glucose, 0.025 *M* Tris chloride, 0.0285 *M* NaCl, 0.008 *M* $MgCl_2$, pH 7.4. The washed nuclei were extracted with 0.1 *M*

Tris Cl–0.003 *M* $MgCl_2$, pH 7.1. This 0.1 *M* Tris T extract was centrifuged at 3,000 g_{av} for 30 min, and the resulting nucleus-free supernate was centrifuged at 105,000 g_{av} for 60 min, yielding sedimented particles of 120–7000 S. The resulting supernate was brought to pH 5.2 with 1.0 *M* acetic acid, and the precipitate collected as nuclear pH 5 enzymes.

Results and Discussion.—Extraction of nuclei: Since 0.1 *M* Tris (hydroxymethyl) aminomethane buffer, pH 7.1 was a more efficient extractant of both proteins and RNA from the nuclei (Table 1) than the neutral 0.1 *M* potassium phosphate buffer used previously.[1, 15, 17] Tris was used as the extracting buffer in all subsequent experiments. Addition of either Ca^{++} or Mg^{++} did not enhance extraction.

TABLE 1

COMPARISON OF NEUTRAL BUFFER EXTRACTIONS OF NUCLEI

	Composition of Extracted Material		
	DNA mgm	Protein mgm	RNA mgm
Whole Nuclei—before extraction	1576	4785	105.5
pH 7.1 Buffer extracts*			
0.1 *M* Sodium phosphate	1.5	680	42.8
0.1 *M* Potassium phosphate	1.3	850	54.4
0.1 *M* Tris chloride	1.6	885	61.8
0.1 *M* Tris Cl–0.003 *M* $CaCl_2$	0.2	720	50.1
0.1 *M* Tris Cl–0.003 *M* $MgCl_2$	0.4	590	41.1

* Unsedimented after centrifugation at 3,000 g_{av} for thirty min.

TABLE 2

EXTRACTION BY NEUTRAL 0.1 *M* TRIS BUFFER OF PROTEIN AND RNA FROM NUCLEI FOLLOWING INCUBATION WITH RADIOACTIVE PRECURSORS

		Alanine-1-C^{14} Incorporation[4]			Adenosine-8-C^{14} Incorporation[5]		
	DNA mgm	Protein mgm	Counts cpm	Spec. Act. cpm/mg	RNA mgm	Counts cpm	Spec. Act. cpm/mgm
Whole Nuclei[1]	1,440	4,220	625,000	148	52.2	131,800	2,520
>7000 S Particles[2]	3.2	...	...	...	5.38	1,380	256
<7000 S Particles[3]							
Extract I	1.94	314	179,000	572	7.09	1,290	182
Extract II	1.52	172	98,200	571	3.20	2,680	837
Extract III	1.20	59	33,600	569	1.81	7,390	4,070
Residual Nuclei	1,428	3,360	218,000	65	33.1	97,200	2,960
Recovery, per cent	99.6	92.5	84.5	...	96.8	83.5	...

Incubations for sixty min. at 37°C with agitation. Approximately 40 mgm (dry weight) of isolated nuclei per ml of incubation medium. Incubation medium of final composition: 0.1875 *M* Sucrose, 0.02 *M* glucose, 0.0285 *M* NaCl, 0.025 *M* sodium phosphate, 0.0065 *M* $MgCl_2$, 0.0015 *M* $CaCl_2$, pH 6.8.

[1] After removal of cytoplasmic fragments soluble in incubation medium.
[2] Sedimented after centrifugation of extracts at 3000 g_{av} for thirty min.
[3] Unsedimented after centrifugation of extracts at 3000 g_{av} for thirty min.
[4] DL-alanine-1-C^{14}, 0.56 μc/0.025 mgm added to each ml of alanine incubation medium.
[5] Adenosine-8-C^{14}, 0.16 μc/0.239 mgm added to each ml of adenosine incubation medium.

Up to 20 per cent of proteins and 60 per cent of RNA could be extracted from the nuclei with only minimal extraction of DNA, the particular yield varying with different preparations.

The yield of both proteins and RNA which were extracted declined from the first extract to the third (Table 2), but, while the specific activity of incorporated radioactivity was almost uniform in the proteins of all three extracts, the specific activity of the RNA of the third extract was almost 30 times that of the first extract (Table 2), suggesting that the third extract contained RNA which had been extracted from the nucleolus.[15]

The time course of alanine-1-C^{14} incorporation into the proteins of the nuclei

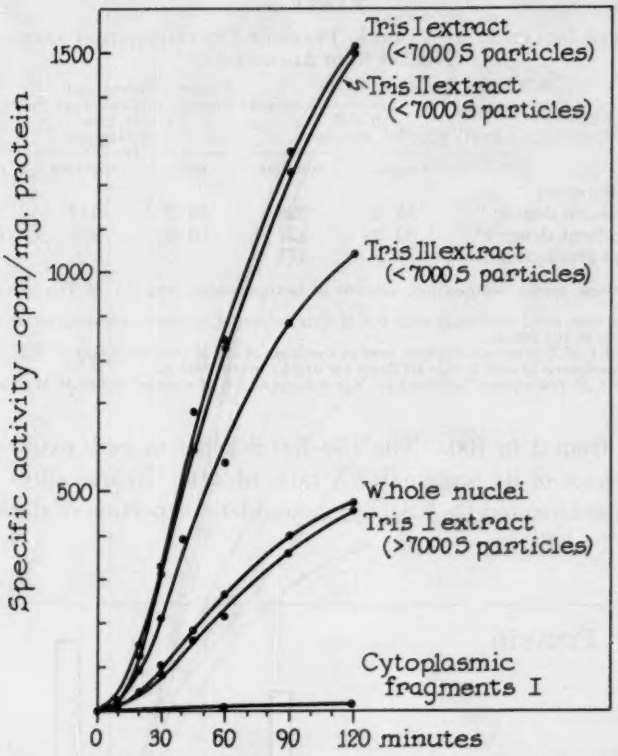

FIG. 1.—Time course of incorporation of alanine-1-C^{14} into proteins of nuclei. Incubation conditions, media composition, and amount of isotope added same as in Table 2.

(Fig. 1) shows a lag period of 10 to 20 min,[1] followed by a progressive increase in labelling. The material which sedimented after centrifugation at 3,000 *g* for 30 min (> 7000 S) resembled the protein of whole nuclei in its specific activity and was discarded. The incubation medium contained cytoplasmic fragments which could be identified by their appearance by electron microscopy; these showed insignificant incorporated radioactivity which was resistant to DNAase preincubation. The proteins of the third extract were always slightly less active than those of the first two extracts (Table 2, Fig. 1).

Centrifugation of extracts: When each of the three Tris extracts of nuclei which had been incubated with alanine-1-C^{14} is ultracentrifuged, an appreciable portion of the extracted proteins and their incorporated radioactivity can be sedimented as ribonucleoprotein pellets (Table 3). Centrifugation in a fixed-angle head is the most convenient method and results in the highest yield of sedimented proteins. As an alternative, centrifugation across a density gradient separates the sedimentable ribonucleoprotein particles away from the less sedimentable soluble proteins in the extracts with which they are present. The particles so separated retain their incorporated radioactivity (Table 3).

When each of the three Tris extracts are subjected to differential ultracentrifugation (Fig. 2), discrete ranges of ribonucleoprotein particles are sedimented, each with its characteristic composition of protein and RNA. Protein/RNA ratios of the

TABLE 3

NUCLEAR PARTICLE ISOLATION BY VARIOUS TYPES OF CENTRIFUGATION AFTER INCUBATION OF NUCLEI WITH ALANINE-1-C^{14}

	Protein Content and Specific Activity of Centrifuge Pellet					
	0.1 *M* Tris extract I		0.1 *M* Tris extract II		0.1 *M* Tris extract III	
	mgm	cpm/mg	mgm	cpm/mg	mgm	cpm/mg
Method of centrifugation						
Fixed angle uniform density[1]	55.4	398	49.2	442	9.8	374
Fixed angle gradient density[2]	30.2	227	16.6	307	5.3	305
Swinging bucket gradient density[3]	28.8	178	...	...	...	...

Incubation conditions, media composition, amount of isotope added, and 0.1 *M* Tris extractions same as in Table 2.

[1] Entire centrifuge tube filled uniformly with 0.1 *M* Tris extract of nucleus. Centrifugation in Spinco Model L No. 40 Head for five hr at 105,000 g_{av}.

[2] Two volumes of 0.1 *M* Tris extract layered over one volume of 1.0 *M* sucrose–0.003 *M* $MgCl_2$ in each centrifuge tube. Centrifuged in Spinco Model L No. 40 Head for five hr at 105,000 g_{av}.

[3] One volume of 0.1 *M* Tris extract layered over one volume of 1.0 *M* sucrose–0.003 *M* $MgCl_2$ in each centrifuge tube.

pellets ranged from 2 to 100. The 150–700 S pellet in each extract is especially interesting because of its protein/RNA ratio of 2/1. Nearly all of the extracted RNA could be sedimented (> 6 S), but a considerable portion of the extracted proteins could not (< 6 S).

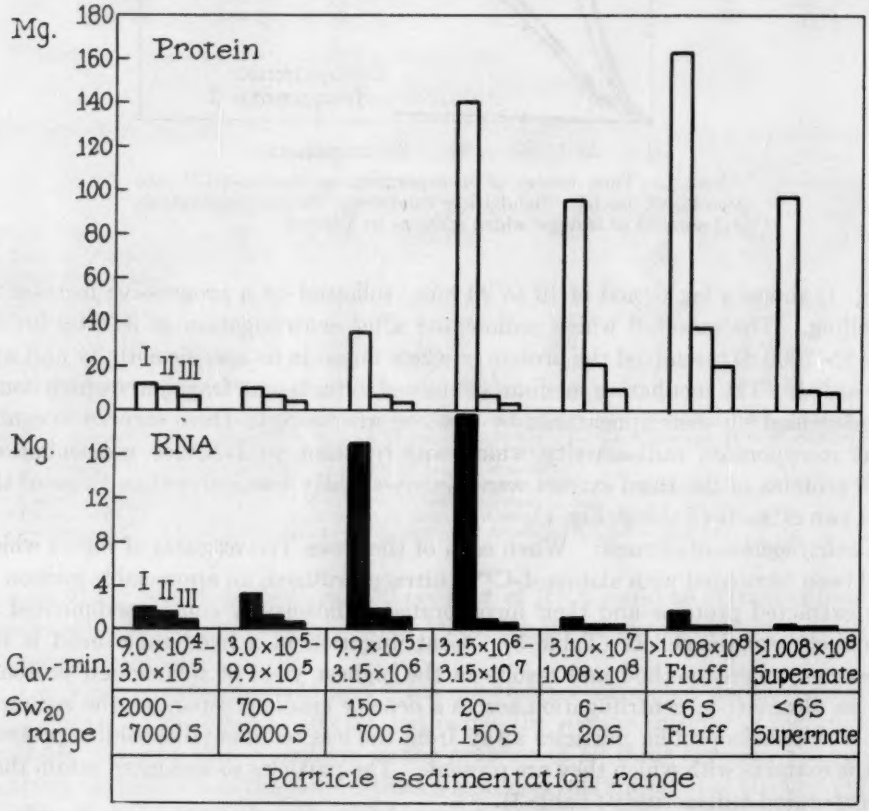

FIG. 2.—Differential ultracentrifugation of nuclear ribonucleoprotein particles. Nuclei extracted successively three times with five volumes of cold 0.1 *M* Tris(hydroxymethyl) aminomethane buffer pH 7.1. Each of three Tris extracts subjected to differential ultracentrifugation as in schedule described in *Materials and Methods*.

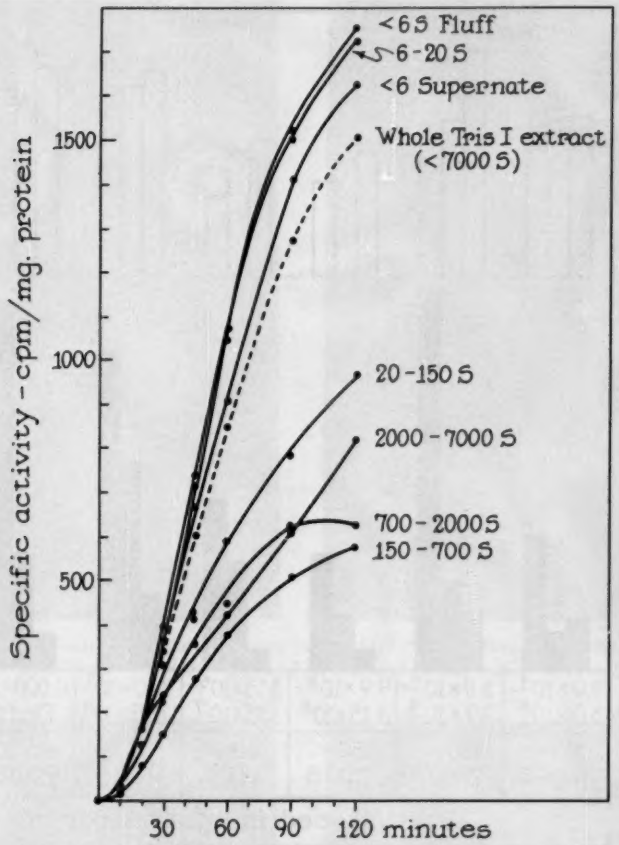

FIG. 3.—Time course of incorporation of alanine-1-C^{14} into proteins of nuclear ribonucleoprotein particles. Incubation conditions, media composition, and amount of isotope added same as in Table 2. Differential ultracentrifugation of 0.1 *M* Tris extract I in schedule described in *Materials and Methods.*

Intranuclear metabolism of particles: The different nuclear ribonucleoprotein particles show marked differences in their rate of incorporation of alanine-1-C^{14} into protein while in the nucleus (Fig. 3). No obvious precursor-product relationships between particles were evident in these simple time-course studies. Because of the known intimacy of the metabolism of protein and RNA in protein synthetic processes in the cytoplasm,[3, 22] it was of interest to study the simultaneous intranuclear metabolism of protein and of RNA in the nuclear ribonucleoprotein particles of one individual. In such a study (Fig. 4), the rates of protein and of RNA metabolism in a given particle range were parallel, being high for both protein and RNA in particles 700–2000 S and particles 6–20 S, and low for both protein and RNA in particles 2000–7000 S, 150–700 S, and 20–150 S.

Both the small amount of supernatant RNA (< 6 S) and the larger amount of supernatant protein (< 6 S) displayed very active intranuclear metabolism within each extract (Fig. 4). The significance of such active metabolism in these compounds is unknown. Preliminary observations suggest that both the carrier RNA and the pH 5.0 enzymes involved in amino acid activation and transfer in the

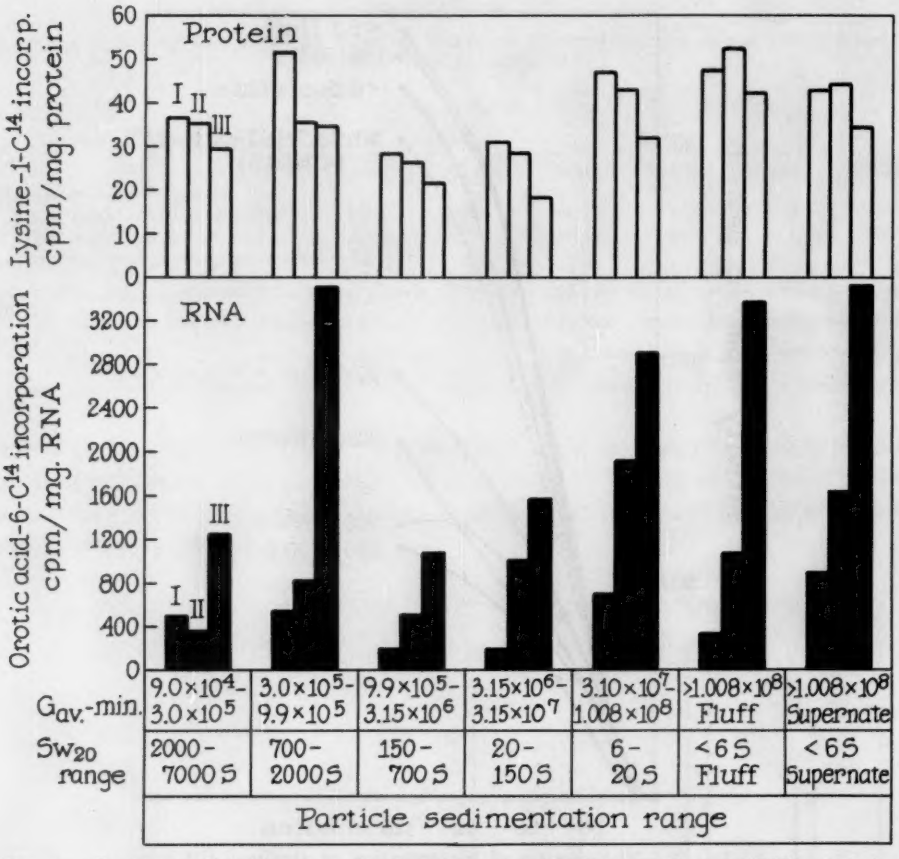

FIG. 4.—Concurrent incorporation of radioactive precursors into proteins and RNA of nuclear particles. Incubation conditions and media composition same as in Table 2. DL-lysine-1-C^{14}, 0.09 μc/0.05 mgm added to each ml of lysine incubation medium. Orotic acid-6-C^{14}, 0.219 μc/0.02 mgm added to each ml of orotic acid incubation medium. Differential ultracentrifugation of 0.1 *M* Tris extracts I, II, III as in schedule described in *Materials and Methods*.

nucleus[23] are sedimentable within 16 hr at 105,000 g_{av},[24] and thus of sedimentation constant greater than 6 S.

Electron microscopy of particles: Electron micrographs of intact isolated nuclei before extraction with neutral buffer reveal the nuclei to be largely free of adherent cytoplasm, only 5 per cent of isolated nuclei showing adherent cytoplasmic fragments. In favorable sections, the nucleolar region of the nucleus can be seen (Fig. 5*a*). Higher magnifications of such intact nuclei reveals the presence of large numbers of granules in both the nucleolus (Fig. 5*b*) and in the extra-nucleolar regions (Fig. 5*c*) of the nucleus. The granules are of two basic types, small solidly dense particles of 15 Å diameter, and slightly larger hollow particles with a dense periphery and a diameter of 25 Å. Both types of particles are found arranged singly, in regular rings of 75–100 Å total diameter, or in irregular clusters of 75 Å diameter. After extraction with neutral buffer, there is an obvious change in the fine structure of the nuclei (Fig. 6), with a decrease in the number of granules, and a coarsening of the remaining nuclear structure.

FIG. 5*a*.—Isolated whole nucleus before buffer extraction. ×15,000.
FIG. 5*b*—Nucleolar region of nucleus in Fig. 5*a*. ×240,000.

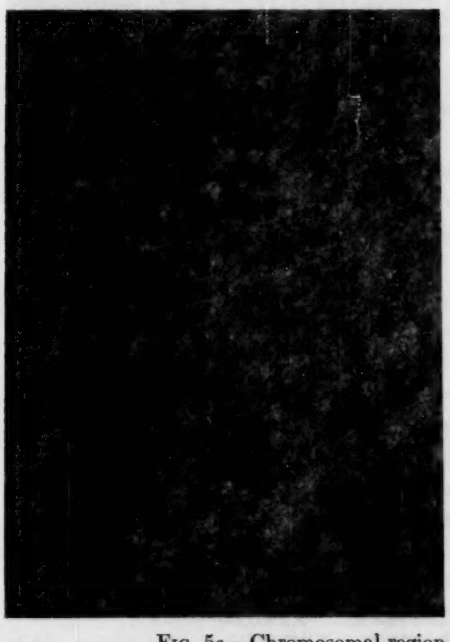

FIG. 5*c*.—Chromosomal region of nucleus in Fig. 5*a*. ×240,000.
FIG. 6.—Isolated residual nucleus after buffer extraction. ×15,000.

Electron micrographs of isolated ribonucleoprotein particles after extraction from the nuclei by neutral buffer reveals the presence of large numbers of dense

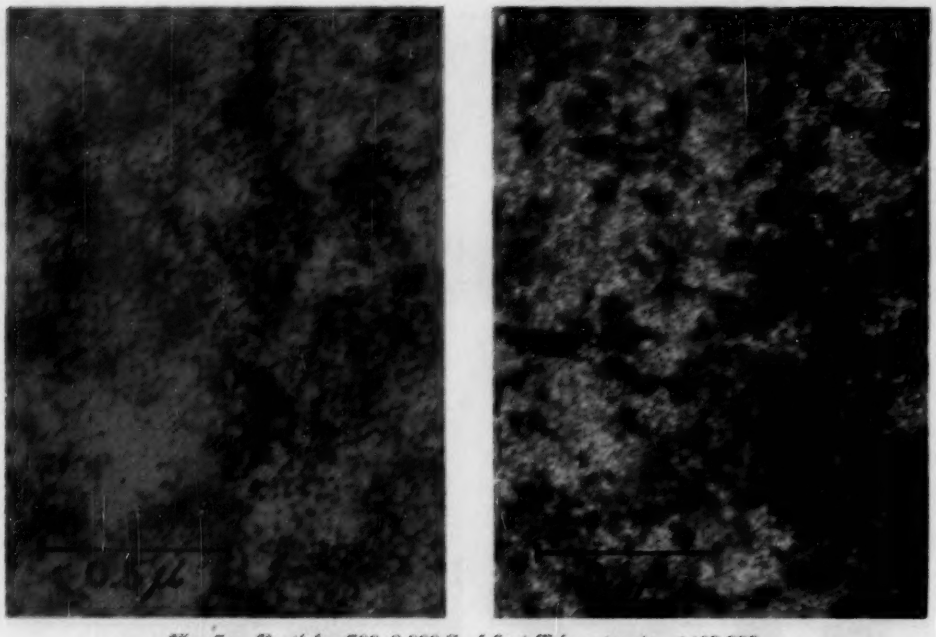

Fig. 7*a*—Particles 700–2,000 S of first Tris extract. ×60,000.
Fig. 7*b*.—Particles 700–2,000 S of the first Tris extract. ×240,000.

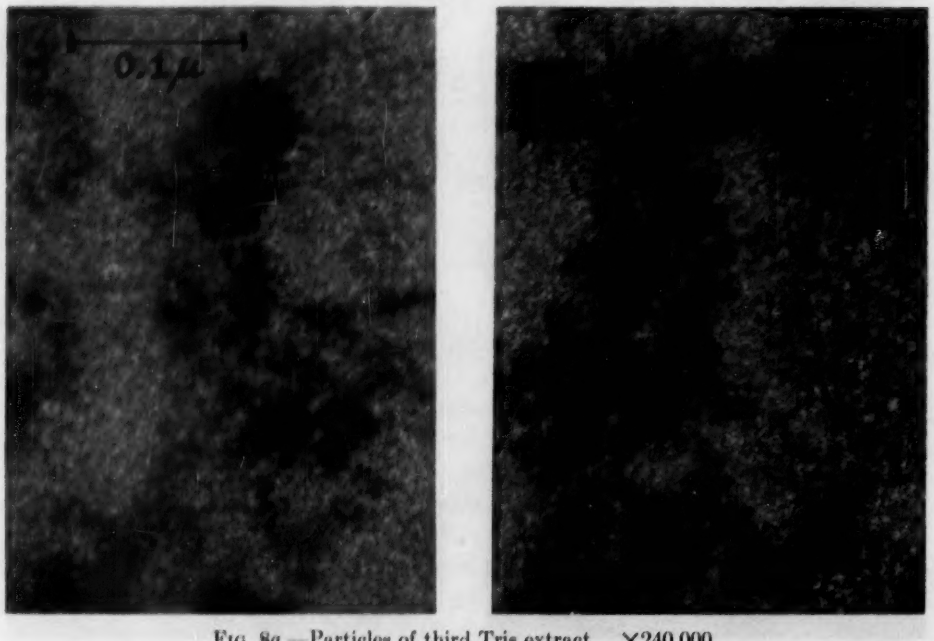

Fig. 8*a*.—Particles of third Tris extract. ×240,000.
Fig. 8*b*.—Particles of third Tris extract. ×240,000.

granules of 100 Å diameter (Fig. 7*a*). Higher magnifications of such granules (Fig. 7*b*) reveals them to be composed of rings and clusters of either hollow particles of 25 Å diameter each. Similar rings (Fig. 8*a*) and clusters (Fig. 8*b*) of 25 Å

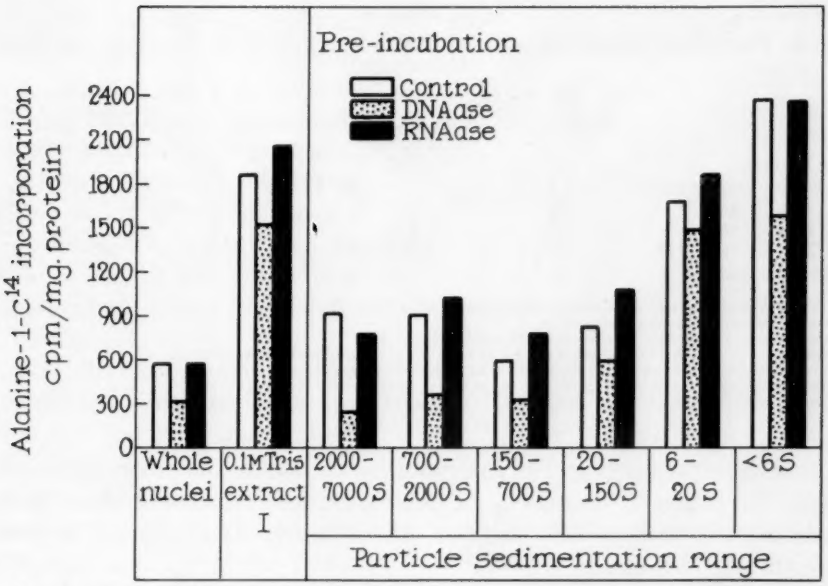

FIG. 9.—Effect of pre-incubation with nucleases on subsequent incorporation of alanine-1-C^{14} into proteins of nuclear particles. Fifteen min pre-incubation of whole nuclei at 37°C in media as described in Table 2, with addition of crystalline pancreatic DNAase 0.25 mgm/ml or of RNAase 0.5 mg/ml, or of neither. 67.5 per cent of DNA removed from whole nuclei by DNAase pre-incubation, and 26.2 per cent of RNA removed from whole nuclei by RNAase pre-incubation. Subsequent incubation with DL-alanine-1-C^{14} as described in Table 2. Differential ultracentrifugation of Tris extracts I as in schedule described in *Materials and Methods*.

diameter solid particles are found in the ribonucleoprotein pellets of both the first and third Tris extracts.

Hollow particles with dense peripheries and a total diameter of 25 Å have been illustrated in the higher magnifications of electron micrographs of elementary chromosomal fibrils (Fig. 9 of ref. 25), of rings of nucleolar particles (Fig. 3 of ref. 7), of nuclear pore annuli granules (Fig. 7 of ref. 12), and of cytoplasmic microsomal granules (Fig. 7 of ref. 12). The possible relationship of such illustrated hollow particles to the ribonucleoprotein particles isolated here is of great interest but is, as yet, undetermined.

Nuclear origin of particles: That the ribonucleoprotein particles are of nuclear and not of cytoplasmic origin can be shown in several ways.

First, it can be shown that in a sodium-supplemented medium as here employed to label the proteins of the nuclei, virtually no labelling of the proteins of the cytoplasmic fragments (Fig. 1, Table 4) occurs, whereas the labelling of the proteins of the nucleus and its particles is brisk.

Second, if the nuclei are pre-incubated with DNAase or with RNAase before incubation with alanine-1-C^{14} (Fig. 9), the rate of incorporation into the proteins of the nuclear particles is found to be sensitive to DNAase preincubation but not to RNAase preincubation. This is in marked contrast to the behavior of cytoplasmic particles, whose protein metabolism is exquisitely sensitive to RNAase preincubation.[3, 4]

Third, when the nuclei are incubated in a potassium environment rather than in

TABLE 4

RATES OF PRECURSOR INCORPORATION INTO PROTEIN AND RNA OF NUCLEAR FRACTIONS AND CYTOPLASM

	Precursor Incorporation into Protein		Precursor Incorporation into RNA	
	lysine-1-C^{14} cpm/mg[3]	alanine-1-C^{14} cpm/mg[4]	orotic acid-6-C^{14} cpm/mg[5]	adenosine-8-C^{14} cpm/mg[6]
Whole nucleus	13	148	5705	2520
Cytoplasmic fragments[1]	1	5	750	244
0.1 *M* Tris Extract I[2]	44	572	269	182
0.1 *M* Tris Extract II[2]	43	571	1215	837
0.1 *M* Tris Extract III[2]	35	569	7570	4070
Residual nucleus	6	65	929	2960

Incubation conditions and media compositions same as in Table 2.
[1] Fraction soluble in incubation medium.
[2] Supernatant after centrifugation at 3,000 g_{av} for thirty min.
[3] DL-lysine-1-C^{14}, 0.09 μc/0.05 mgm added to each ml of lysine incubation medium.
[4] DL-alanine-1-C^{14}, 0.56 μc/0.025 mgm added to each ml of alanine incubation medium.
[5] Orotic acid-6-C^{14}, 0.219 μc/mgm added to each ml of orotic acid incubation medium.
[6] Adenosine-8-C^{14}, 0.16 μc/0.239 mgm added to each ml of adenosine incubation medium.

the usual environment (Fig. 10), incorporation of alanine-1-C^{14} into the proteins of the particles is greatly decreased. This is in marked contrast to the behavior of cytoplasmic ribonucleoprotein particles, whose protein metabolism is increased by potassium and decreased by sodium.[2]

Fourth, the particles obtained in the first Tris extract are composed of protein of

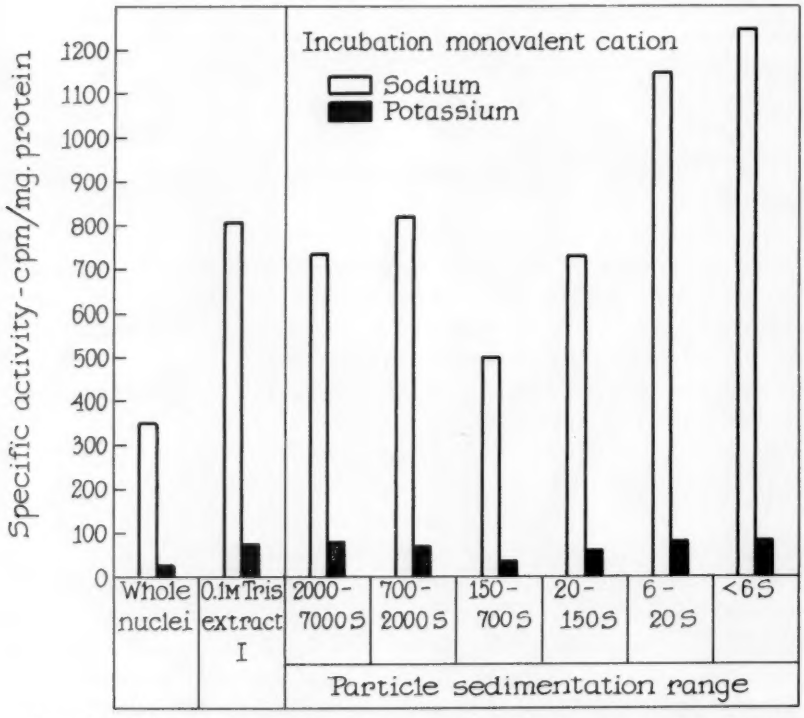

FIG. 10.—Effect of monovalent cation on incorporation of alanine-1-C^{14} into proteins of nuclear particles. Incubation conditions, isotope added, and media same as in Table 2 except in the potassium environment incubation, equimolar amounts of KCl, and potassium phosphate pH 6.8 were substituted for usual NaCl and sodium phosphate pH 6.8. Differential ultracentrifugation of Tris extracts I as in schedule described in *Materials and Methods*.

high specific activity and RNA of low specific activity. The specific activity of this RNA is, in fact, lower than that in the cytoplasmic fragments contained in the incubation medium (Table 4), suggesting that the RNA of the incubation medium cannot be contributing by contamination to the very low specific activity of the RNA of the first Tris extract.

Finally, the electron micrography described above reveals that the solid and hollow particles of 15–25 Å diameter extracted by neutral buffer from the nuclei resemble both in size and arrangement the particles seen in large numbers in the intact nuclei before extraction and in decreased numbers in the nuclei after extraction.

Metabolism of particles in a nucleus-free system: When isolated nuclear ribonucleoprotein particles are incubated in a nucleus-free system, they are found to be capable of active incorporation of leucine-1-C^{14} into the proteins of the isolated particles (Fig. 11). A study of the requirements for such incorporation in a nucleus-free system (Table 5) shows the process to require the nuclear pH 5 enzymes, adenosine triphosphate (ATP), guanosine triphosphate (GTP), and a source of generating ATP. Cytidinetriphosphate (CTP) was slightly inhibitory to the process. The system contained only trace amounts of measureable DNA.

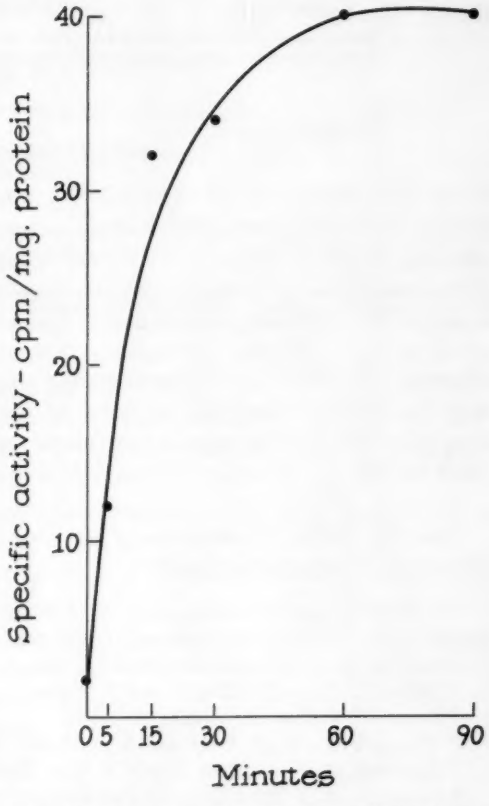

FIG. 11.—Incorporation of Leucine-1-C^{14} into proteins of isolated nuclear ribonucleoprotein particles in a nucleus-free system. Isolation of nuclei, nuclear ribonucleoprotein particles 120–7,000 S, and nuclear pH 5 enzymes <120 S as described in *Materials and Methods* for studies on a nucleus-free system. The complete system consisted of: nuclear ribonucleoprotein particles 120–7,000 S (5.5 mgm protein, 1.23 mgm RNA, and <0.04 mgm DNA), nuclear pH 5 enzymes <120 S (0.96 mgm protein, 0.04 mgm RNA, and <0.001 mgm DNA), 0.20 mgm pyruvate kinase, 20 μ moles sodium phosphoenolpyruvate (PEP), 2.0 μ moles sodium ATP, 1.0 μ mole of sodium GTP and of sodium CTP, and L-leucine-1-C^{14} (2.0 μc/0.05 mgm) in a final volume of 2.0 ml of an incubation medium of following composition: 0.1875 *M* sucrose, 0.02 *M* glucose, 0.025 *M* Tris Cl, 0.0285 *M* NaCl, 0.008 *M* $MgCl_2$, pH 7.4. Incubated at 37°C in Air.

Summary.—Ribonucleoprotein particles can be extracted by neutral buffer solutions from isolated nuclei of calf thymus lymphocytes. Such particles can be fractionated and characterized by their ease of extraction, their sedimentability, their protein and RNA composition, and by their rate of protein and RNA metabolism in the nucleus. The rates of protein and of RNA metabolism in the nucleus are parallel in each class of particles. The particles are revealed by electron micrography to consist of elementary units of 15–25 Å diameter, arranged in rings or

TABLE 5

REQUIREMENTS FOR INCORPORATION OF LEUCINE-1-C^{14} INTO PROTEINS OF ISOLATED NUCLEAR RIBONUCLEOPROTEIN PARTICLES IN A NUCLEUS-FREE SYSTEM[1]

	cpm/mgm Protein
Complete system	62
" " minus Nuclear Particles	9
" " " Nuclear pH 5 Enzymes	33
" " " PEP	20
" " " ATP	27
" " " GTP	26
" " " CTP	68

[1] Isolation of nuclei, nuclear ribonucleoprotein particles 120–7000 S, and nuclear pH 5 enzymes <120 S as described in materials and methods for studies on a nucleus-free system. Incubation medium, isotope, and additions as in Figure 11, except for deletions as indicated. Incubated 37°C 60 min in air.

clusters. Metabolic evidence suggests that certain of the classes of isolated particles may be derived from the nucleolus of the nucleus. Isolated particles extracted from the cell nucleus remain capable of incorporating radioactive precursors into their constituent proteins in a nucleus-free system.

The able technical assistance of Mr. Don Jasper in the preparation of the electron micrographs was appreciated.

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EVIDENCE FOR HELICAL STRUCTURE IN POLYURIDYLIC ACID

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Most of the homopolymers of ribonucleotides thus far prepared with polynucleotide phosphorylase[1] have been found to interact with themselves and assume multistranded helical configurations under certain conditions. In acid solution, polyadenylic acid (poly A)[2] and perhaps poly C[3] form helical two-stranded structures. Poly I has been reported to form a three-stranded helix.[4] No data are available on poly G, which is very difficult to polymerize with the enzyme, or on polyribothymidylic acid, recently synthesized by Griffin *et al.*[5] Poly U has been regarded as a polynucleotide which does not undergo a helix-coil transition. Evidence is here presented indicating that poly U, too, can form an ordered, hydrogen-bonded structure.

Formation of known helical polynucleotide structures is accompanied by a marked hypochromicity.[2-4] When dilute salt solutions of poly U are cooled to 2°C, such effects are noted. As may be seen in Figure 1, in 0.01 *M* $MgCl_2$ at neutral pH, poly U undergoes a 30 per cent drop in extinction upon cooling to 2°C. The ultraviolet absorption maximum shifts from 260 mμ to 258.5 mμ, the minimum from 230 mμ to 234 mμ. Divalent cations produce these changes much more effectively than monovalent ones. For example, sodium chloride at a concentration of 1 *M* produces approximately the same hypochromicity, namely, 25 per cent, as $MgCl_2$ at 10^{-3} *M*.

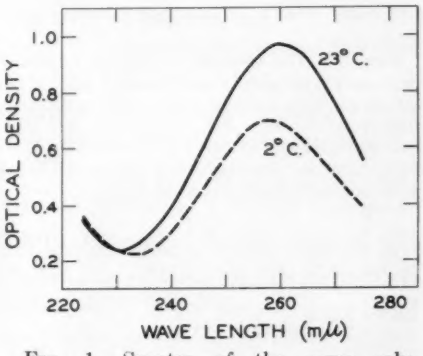

FIG. 1.—Spectra of the same solution of poly U in 0.01 *M* $MgCl_2$, pH 7.4, at two temperatures.

When a chilled poly U solution is warmed, there is an abrupt rise in absorption similar to those described during the thermal conversion of poly I + I + I or poly A + U to the random state[6] (Fig. 2). The transition temperature (T_m) in 0.01 *M* $MgCl_2$, pH 7.4, is 5.8°C, the point of greatest slope of the curve. The baseline for completely interacted poly U was apparently not reached. In 0.001 *M* $MgCl_2$ the T_m is lowered to approximately 3.3°C.

As solutions of polymers in helical form are heated, the $(\alpha)_D$ decreases by approximately 300°, to less than 25°, and the midpoint of this transition corresponds to the T_m determined by following absorption changes with temperature.[6] In the case of poly U, the $(\alpha)_D$ is found to be 290° at 0.5°C and decreases to 11° at 23°C. The rate of change with temperature is greatest at 5.8°C. The mercury line at 365 mμ is far more sensitive than the conventional sodium line, and yields a rotation (1%, 0.5°C) of 1350° (Fig. 2).

The fact that this transition occurs over a narrow temperature range indicates the "melting" of a regular cooperative structure rather than a gradual breaking of

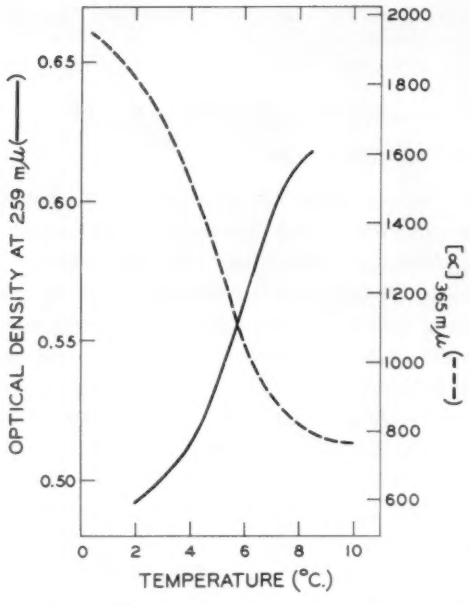

FIG. 2.—The change in optical density (solid line) and optical rotation (dashed line) of poly U solutions with temperature. The poly U is dissolved in 0.01 *M* $MgCl_2$, adjusted to pH 7.4. The concentration of the polymer for the optical rotation curve is approximately 25 times that of the solution used for the optical density curve.

random intrachain base associations. The latter, observed, for example, on heating of neutral solutions of poly A, extends over a very broad temperature range and produces over-all changes in $[\alpha]_D$ on the order of 150 to 200°.[7]

The weakness of the self-bonding of poly U is indicated by the very low T_m, as compared with about 40°C for poly I + I + I and over 70° for poly A + U, both in 1 *M* NaCl at pH 6.[6] Further, a comparison of the interaction of poly U with poly A at 25° and at 2°C shows no significant differences in rate or extent of interaction. This indicates again that the affinity of poly A for poly U is much stronger than the affinity of poly U for itself. Hence poly A can compete very successfully for unbonded poly U, and displaces the equilibrium in favor of poly A + U

In summary, it appears that poly U exhibits the same hypochromicity, spectral shifts, and changes in optical rotation which have been described for other synthetic polyribonucleotides as typical of formation of helical structures, and which have been proved by X-ray crystallography to correspond with the existence of helical structure in the cases of poly A and poly I. The final proof for a helical structure for poly U at low temperatures must, of course, also come from X-ray analysis, and attempts are being made in this direction.

The author wishes to express her appreciation to Dr. Leon Heppel for his encouragement and helpful discussion during the course of this work.

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*INHIBITION OF MAMMALIAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY STEROIDS**

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It has been considered for some time that steroid hormones may exert their regulatory effects by influencing certain enzymes.[1] However, our present knowledge of the mechanisms of steroid action upon enzymes is limited. Estrogens have been shown to stimulate the transfer of hydrogen between di- and triphosphopyridine nucleotides,[2, 3] although there is disagreement as to the role of steroids in this reaction.[4, 5] Recently, steroids were found to inhibit reduced diphosphopyridine nucleotide oxidation by enzyme preparations from mammalian and microbial sources.[6, 7] This action appears to be a general property shared by a large number of steroids.[7]

This study demonstrates a highly specific inhibitory effect on mammalian glucose-6-phosphate dehydrogenase (G-6-PD) of very low concentrations of dehydroisoandrosterone, pregnenolone, and certain related steroids. These steroids are not inhibitors of yeast or spinach G-6-PD or of mammalian 6-phosphogluconic dehydrogenase or isocitric dehydrogenase.

Materials and Methods.—Preparation of enzymes: G-6-PD was purified from human erythrocytes by a procedure described in detail elsewhere[8] involving ammonium sulfate fractionation followed by absorption on calcium phosphate gel, elution in a phosphate buffer, and a second series of ammonium sulfate precipitations. The final product generally had a specific activity 500-fold that of the crude hemolysate in a yield of about 40 per cent. This preparation was essentially free of hemoglobin and 6-phosphogluconic dehydrogenase activity.

Crude enzyme preparations were obtained from human and rat tissues and from spinach by homogenizing freshly obtained material in isotonic potassium chloride buffered at pH 7.4 (10%, weight/volume). The homogenate was centrifuged 30 minutes at 18,000 $\times$ g to sediment debris. The supernatant fluid was employed for assays of enzyme activity.

Partially purified yeast G-6-PD and pig heart isocitric dehydrogenase were obtained from Sigma Chemical Corporation. Partially purified 6-phosphogluconic dehydrogenase was prepared from rat liver by the method of Glock and McLean.[9]

Enzyme assays: Purified preparations of G-6-PD were assayed by the method of Kornberg and Horecker.[10] In measuring G-6-PD activity in crude tissue preparations, due to the presence of 6-phosphogluconic dehydrogenase, the rate of TPNH formation exceeds the rate of glucose-6-phosphate oxidation. Accordingly, in crude preparations, G-6-PD activity was assayed by the method of Kornberg and Horecker[10] determining the initial rate of TPNH formation by measuring the change of absorption at 340 mμ with an automatic recording Cary spectrophotometer or, alternatively, by the method of Glock and McLean.[9] In this latter method, G-6-PD activity is taken as the difference in the rate of TPNH reduction when the reaction mixture contains both 6-phosphogluconate plus glucose-6-phosphate and in the presence of 6-phosphogluconate alone. Comparable results with

regard to the effects of steroids were obtained with both methods of assay of G-6-PD activity. 6-Phosphogluconic dehydrogenase activity was determined by the method of Horecker and Smyrniotis.[11] Isocitric dehydrogenase activity was assayed by the method of Ochoa.[12] The steroids were added in 0.02 ml of dioxane. The total volume of the reaction mixture was 2.5 ml. Determinations of enzyme activity in the absence of steroids were performed in identical reaction mixtures containing 0.02 ml of dioxane. Addition of steroids in propylene glycol yielded results similar to those obtained when dioxane was used as the diluent.

Sources of steroids: All steroids were obtained from commercial sources with the following exceptions: dehydroisoandrosterone, androsterone, etiocholanolone, Reichstein's Compound "S," desoxycorticosterone and 17-hydroxypregnenolone were kindly supplied by Dr. Seymour Lieberman, and 3α-OH-Δ^5-androstene-17-one, epiandrosterone and androstenedione were kindly supplied by Dr. Michael Lombardo.

TABLE 1

EFFECT OF STEROIDS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASES

Compound Concentration (M)	Purified Human RBC G-6-PD			Crude Rat Adrenal G-6-PD		
	4×10^{-5}	1×10^{-6}	4×10^{-7}	4×10^{-5}	1×10^{-6}	4×10^{-7}
	% Inhibition*			% Inhibition		
Pregnenolone	70	23	13	73	30	23
17-OH-Pregnenolone		21	8		21	
3β-Hydroxyallopregnan-20-one	61	13	3	78	20	10
Pregnan-3,20-dione	47	9	0	41	0	
17α-OH-21-acetoxy-pregnenolone	19†	0			0	
Pregnenolone-3-methyl-ether	19	0			0	
Progesterone	23	0		3	0	
17α-OH-Progesterone	18	0				
Dehydroisoandrosterone	80	28	18	90	45	21
3α-OH-Δ^5-androstene-17-one	80	30	15	90	41	18
Epiandrosterone	85	34		95	45	
Androstane-3,17-dione	75	15				
Δ^4-Androstene-3,17-dione	55	10	0	52	3	0
Androsterone	52	11	0	50	10	0
Etiocholanolone	50	10	0	60	20	
Estrone	21†	0		15*	0	
Stilbesterol	8	0		5	0	

* Per cent inhibition of enzyme activity is expressed in terms of the rate of the reaction in the presence of the steroid compared to that in the absence of the steroid. The steroid was added to the reaction mixture in 0.02 ml of dioxane and all controls were run with 0.02 ml of dioxane.

† These compounds were insoluble at 4×10^{-5} M and the data are for the effect of the steroid at a concentration of 1×10^{-5} M.

Results.—G-6-PD, purified from human red cells or in crude preparations of rat adrenal, is inhibited by pregnenolone and other C_{21} steroids, 17α-hydroxy-pregnenolone, 3β-hydroxyallopregnan-20-one, pregnan-3,20-dione, and by dehydroisoandrosterone, and other C_{19} steroids, 3α-OH-Δ^5-androstene-17-one, epiandrosterone, androstane-3,17-dione, Δ^4-androstene-3,17-dione, androsterone and etiocholanolone, at 1×10^{-6} M (Table 1). Certain of these steroids had inhibitory effects at 10^{-7} M (Tables 1 and 2).

The following steroids were tested and found not to inhibit G-6-PD at concentrations as high as 4×10^{-5} M: estradiol-17α, estradiol-17β, estriol, hydrocortisone, cortisone, prednisolone, desoxycorticosterone, cortexolone (Compound "S"), cholesterol, stigmasterol, Na tauracholate, cyclohexanone, cyclopentanone, Δ^5-androstene-3,17-diol, 17α-methyl-Δ^5-androstene-3β,17β-diol-17-benzoate, testos-

TABLE 2

EFFECT OF DEHYDROISOANDROSTERONE AND PREGNENOLONE ON DEHYDROGENASES OF VARIOUS SOURCES

Enzyme*	Source	Dehydroisoandrosterone 1×10^{-5} M % Inhibition†	Dehydroisoandrosterone 1×10^{-7} M % Inhibition†	Pregnenolone 1×10^{-5} M % Inhibition	Pregnenolone 1×10^{-7} M % Inhibition
G-6-PD	Purified RBC—human	60	10	58	5
	Crude RBC—human	69	16	55	10
	Crude liver—human	82	20	56	10
	Crude adipose—human	42	0	35	0
	Crude adrenal—rat	80	10	55	5
	Crude liver—rat	78	10	50	5
	Crude testes—rat	85	20	71	10
	Purified yeast	0		0	
	Crude spinach	0		0	
6-PGD	Crude RBC—human	0		0	
	Crude adipose—human	0		0	
	Purified liver—rat	0		0	
	Crude adrenal—rat	0		0	
	Crude liver—rat	0		0	
	Crude testes—rat	0		0	
	Crude spinach	0		0	
ICD	Crude adipose—human	0		0	
	Purified heart—pig	0		0	
	Crude adrenal—rat	0		0	
	Crude liver—rat	0		0	
	Crude testes—rat	0		0	

* Abbreviations: G-6-PD, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconic dehydrogenase; ICD, isocitric dehydrogenase; RBC, red blood cell. For details of sources of enzymes see text.

† Per cent inhibition of enzyme activity is expressed in terms of the rate of the reaction in the presence of the steroid compared to that in the absence of the steroid. The steroid was added to the reaction mixture in 0.02 ml of dioxane and all controls were run with 0.02 ml of dioxane.

terone, pregnan-3α,20α-diol, ethisterone, 16,17-epoxy-21-acetoxy pregnenolone, 16,17-oxido-pregnenolone acetate, and pregnan-17α21-diol-3,11,20-trione-21-acetate.

In addition to the effect of steroids on G-6-PD indicated in Table 1, dehydroisoandrosterone and pregnenolone were examined for their inhibitory action against a variety of G-6-PD from other sources and against two other dehydrogenases catalyzing reactions involving TPN reduction. It was found that the steroids inhibited G-6-PD from several human and rat tissues, but not from spinach or yeast and not 6-phosphogluconic or isocitric dehydrogenases from any of the several tissues examined (Table 2).

To determine whether dehydroisoandrosterone and pregnenolone are competitive or noncompetitive inhibitors of G-6-PD, the activity of the purified preparation of G-6-PD was measured in the absence and presence of the steroid over a wide range of substrate concentrations. These experiments were performed with an excess of TPN and limiting concentrations of glucose-6-phosphate on the one hand, and, with an excess of glucose-6-phosphate and limiting concentrations of TPN on the other hand[13] (Fig. 1). The data indicate that the steroids are noncompetitive inhibitors of G-6-PD with respect to either of its substrates, TPN or glucose-6-phosphate. The reciprocal plots of the enzyme activity in the presence of a constant amount of inhibitor resulted in straight lines which appear parallel to that obtained in the absence of inhibitor. This suggests that the steroid inhibition of G-6-PD may be an example of anti-competitive inhibition.[14, 15]

The degree of inhibition of G-6-PD by steroids is not related to the order of addition of the reactants. Thus, the same inhibition (70 to 80 per cent) was found when

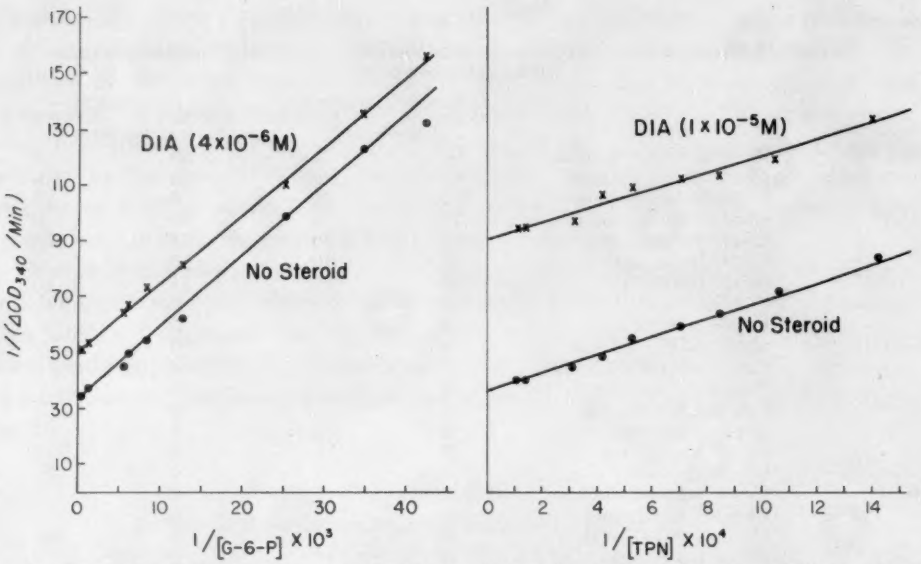

FIG. 1.—Relation between 1/v and 1/(S) for purified RBC glucose-6-phosphate dehydrogenase in the absence and in the presence of a constant amount of dehydroisoandrosterone. In the study plotted on the left, the concentration of glucose-6-phosphate was varied, and in the study plotted on the right, the concentration of TPN was varied. The steroid was added to the reaction mixture in dioxane. Studies without steroid were performed in reaction mixtures containing dioxane.[16]

G-6-PD was preincubated for 15 minutes at 25°C. with dehydroisoandrosterone or pregnenolone (1×10^{-5} *M*) in the presence or absence of TPN and/or glucose-6-phosphate.

The enzyme preparations used did not catalyze a TPN linked oxidation or reduction of the steroids employed. There was no detectable change in the level of TPNH or TPN, as measured spectrophotometrically, when the nucleotides, steroids and enzymes were combined. In addition, substitution of TPNH for TPN in the reaction mixture, in the presence of dehydroisoandrosterone, pregnenolone, or related steroids at 1×10^{-5} *M*, was associated with no change in optical density measured at 340 $m\mu$.

Discussion.—Dehydroisoandrosterone, pregnenolone, and certain related steroids have been found to be potent inhibitors of mammalian G-6-PD catalyzed TPNH formation. The action of these steroids has a specificity with regard to both the dehydrogenase inhibited and the structure of the inhibiting compound. Thus, G-6-PD of yeast and spinach and mammalian 6-phosphogluconic and isocitric dehydrogenases are not inhibited. All the steroids with an inhibitory action on G-6-PD possess a ketone group in the C_{17} or C_{20} positions. Progesterone, corticosteroids, estrogens, and steroids with a hydroxyl rather than a ketone group at C_{17} or C_{20} have relatively little and, generally, no inhibitory effect on G-6-PD. On the other hand, the presence of a ketone or an α or β hydroxyl group at C_3 or a saturated or unsaturated bond at C_{4-5} or C_{5-6} have little effect on the inhibitory action of the steroids tested. In addition, the steroid effect appears to be a catalytic one, since low concentrations of steroid inhibited the reduction of relatively large amounts

of TPN. Thus, as inhibitors of G-6-PD, these steroids have several characteristics compatible with a biologically significant enzyme inhibitor.

Recently, McKerns and Bell[17] reported that stilbesterol and, to a lesser extent, estrone, estradiol, pregnenolone, and progesterone, inhibited G-6-PD of rat or cow adrenal. The present results differ from these in several respects. The relative effects obtained with the compounds tested differ widely. Further, no preincubation was needed to obtain the steroid inhibition of G-6-PD in the present work. Preincubation of the enzyme preparations in the presence of the steroid for periods up to one hour did not enhance the inhibitory effects of the several compounds tested.

It has been demonstrated that some steroids may catalyze a pyridine-nucleotide transhydrogenation.[2-5] The presently described steroid inhibition of G-6-PD catalyzed TPN reduction is apparently unrelated to any such enhancement of TPNH oxidation. It is also unlikely that this steroid effect reflects a reaction with TPN, in view of the failure of the steroids to inhibit 6-phosphogluconic or isocitric dehydrogenases in the same preparations in which G-6-PD was markedly affected.

Our limited knowledge of the metabolism of the several steroids which inhibit G-6-PD makes it difficult to predict the possible physiological consequences of this steroid action. Nevertheless, it is reasonable to consider that such action of dehydrosterone, pregnenolone, and related steroids might function as a regulatory mechanism of significance in carbohydrate metabolism. Of particular pertinence to steroid metabolism is the generation of TPNH.[18] There is much evidence to indicate that TPNH is required at several steps in steroid biosynthesis, including the conversion of squalene to cholesterol,[19, 20] the conversion of cholesterol to pregnenolone,[21] steroid hydroxylations,[22-25] the conversion of androgen to estrogens[26] and cholesterol side chain cleavage.[27] The metabolic function of dehydroisoandrosterone is not well understood.[28] It is secreted by the adrenals in significant amounts and is believed to be the major precursor of the urinary 17-ketosteroid. The suggestion has been made that dehydroisoandrosterone is an intermediate in adrenal androgen synthesis.[29] More data[30, 31] are available on the biochemical role of pregnenolone. It appears that this steroid is a precursor common to progesterone, androgens, estrogens, and corticoid hormones and is derived, in part at least, from cholesterol. It has been suggested that the rate limiting step in the conversion of cholesterol to steroid hormones is involved in cholesterol transformation to pregnenolone.[21, 32, 33]

Inhibition of TPNH generation by pregnenolone or dehydroisoandrosterone could function as a control mechanism in the formation of these key intermediates in steroid hormone synthesis. For the steroid inhibitor of G-6-PD to effect a regulatory action of this type implies further that "intracellular geometry" is such that TPNH generated in the pentose phosphate pathway, but not that in the tricarboxylic acid cycle, is required for the rate limiting step in the synthesis of these intermediate steroids.

Summary.—Dehydroisoandrosterone, pregnenolone, and certain related steroids, at concentrations of 1×10^{-6} *M* or less, inhibit mammalian glucose-6-phosphate dehydrogenase activity. These steroids do not inhibit 6-phosphogluconic or isocitric dehydrogenases or spinach or yeast glucose-6-phosphate dehydrogenase. A variety of other steroids, including estrogens, testosterone, corticosteroids, and

progesterone had little or no effect on glucose-6-phosphate dehydrogenase in concentrations as high as 4×10^{-5} *M*.

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STRAND SEPARATION AND SPECIFIC RECOMBINATION IN DEOXYRIBONUCLEIC ACIDS: BIOLOGICAL STUDIES

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It is clear that the correlation between the structure of deoxyribonucleic acid (DNA) and its function as a genetic determinant could be greatly increased if a means could be found of separating and reforming the two complementary strands. In this and the succeeding paper[1] some success along these lines is reported. This paper will deal with the evidence provided by employing the transforming activity of DNA from *Diplococcus pneumoniae* while the succeeding paper[1] will summarize physical chemical evidence for strand separation and reunion.

Bacterial transformation offers a unique approach to this problem since the activity of DNA isolated from genetically marked strains can be assayed after being subjected to various treatments. We have concentrated on thermal treatment to accomplish our goal for several reasons. First, the accumulated experience in this Laboratory[2–5] has shown that exposure of DNA to carefully controlled temperatures for certain periods of time can denature the DNA molecules with minimal damage to their chemical structure; this is a prerequisite to strand separation and furthermore the ease of precise control of the temperature offers an obvious means of providing nearly reversible conditions which would optimize the chances of reuniting the DNA strands. Moreover, it has been shown in one case that thermal treatment did lead to strand separation[6] and there is considerable evidence that transforming activity falls sharply in the region of thermal denaturation.[5, 7, 8] By utilizing these observations and by giving particular attention to the *rate* of cooling from the elevated temperature we have been able to demonstrate a pattern of inactivation and restoration of biological activity consistent with strand separation and specific reunion. It appears that this reunion can take place between complementary strands or between strands of two closely related molecules from mutant strains of *D. pneumoniae*.

Experimental Details.—The strains of *D. pneumoniae* and the transformation techniques employed have been described previously.[9–11] The isolation of DNA from *D. pneumoniae* and other bacterial species was carried out by a procedure described elsewhere.[12] The samples of pneumococcal DNA had sedimentation constants, $s_{20,w}$ measured in 0.15 *M* NaCl plus 0.015 *M* sodium citrate in the range of 24 to 26 *S* (corresponding to molecular weights of 8–10 million).

In most experiments pneumococcal DNA was dissolved in 0.15 *M* NaCl plus 0.015 *M* sodium citrate (hereafter referred to as standard saline-citrate) and heated in small tubes or flasks by immersion in boiling water. When *fast cooling* was employed, samples were transferred to tubes precooled in ice water. In cases where *slow cooling* was desired, samples were placed in an insulated water bath (six liters) at the elevated temperature, and allowed to cool with the heaters turned off. In a typical experiment the drop in temperature took the following time course: 89°—0 (minutes), 80°—15, 70°—40, 60°—75 and 50°—130. The relationship between concentration and transforming activity of DNA which has been heated at 100°

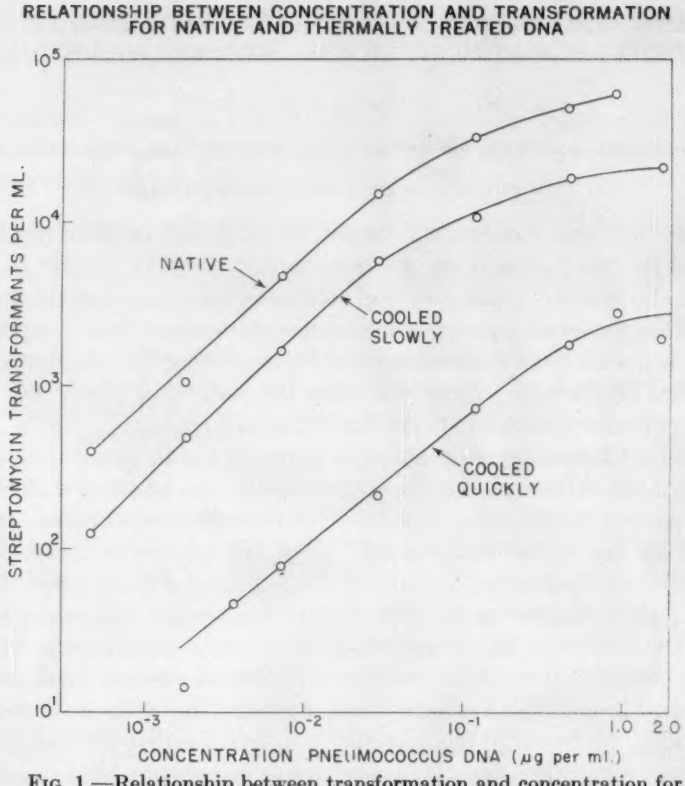

FIG. 1.—Relationship between transformation and concentration for native and thermally denatured DNA (cooled quickly and slowly).

Pneumococcal DNA was heated at 100° for 10 minutes in standard saline-citrate. Hot concentrated saline-citrate was added to a final concentration of 0.3 *M* NaCl and 0.03 *M* Na citrate and a DNA concentration of 20 μg/ml. The mixture was subdivided into two portions, one cooled quickly, the other slowly from 90° to room temperature. The native DNA was an unheated aliquot of the same preparation.

and then cooled quickly and slowly, as well as native unheated DNA is shown in Figure 1. All assays were carried out in the linear portion of the transformation-concentration curve.

Results.—Thermal denaturation and marker inactivation: The thermal denaturation of DNA can be followed by changes in the absorbance at 2600 A. observed either as a function of the temperature of the solution itself or the temperature to which it has been heated prior to cooling to room temperature (25°). The difference between these two temperature profiles results from reformation of base pairs as the temperature is lowered; such reformation has been thought to be nonspecific, involving short regions of chains that are not exactly complementary.[1, 3] The transforming activity, as measured by the ability of the heated pneumococcus DNA to effect transformation of wild-type pneumococcus with respect to three unlinked resistance markers: streptomycin, erythromycin, and bryamycin (which behaves as a high-level micrococcin resistance marker), was followed as a function of temperature. The results together with the absorbance-temperature profiles are shown in Figure 2. From this it is clear that different markers have different ther-

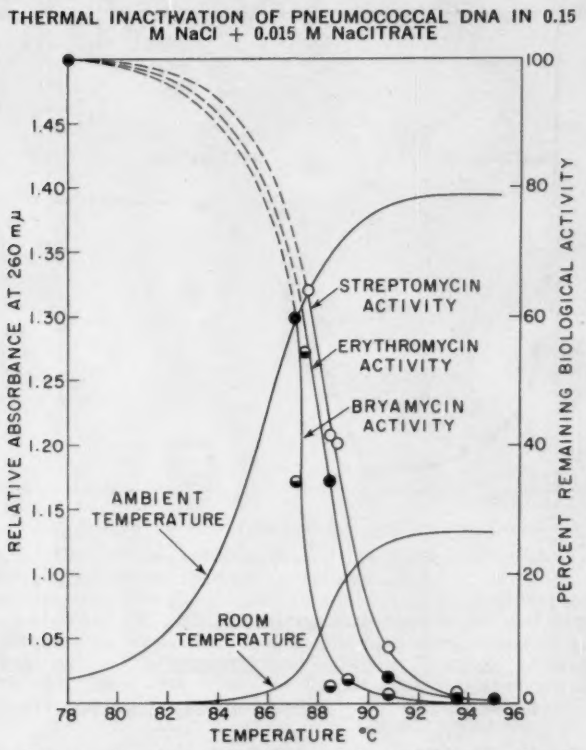

FIG. 2.—Loss in transforming activity and denaturation of DNA as a function of temperature.

Pneumococcal DNA was heated at 20 μg per ml in standard saline-citrate. The absorbance at 260 mμ was recorded for a series of temperatures both at the ambient temperature (after correction for thermal expansion) and after cooling (in air) to room temperature. After each cooling a sample was removed, diluted, and assayed for its ability to transform with respect to streptomycin, erythromycin, and bryamycin.

mal resistance in agreement with the results of others.[7, 8] This difference in sensitivity is most likely related to differences in base composition among the individual DNA molecules in which the markers are located.[5] It is important to note, however, that the thermal inactivations though differing one from another, are much more closely related to the absorbance-temperature curve determined at room temperature than to the one obtained at the ambient temperatures. Hence, the possibility exists that some helical regions melted out at the elevated temperature and have reformed on cooling with return of biological activity.

A stronger suggestion that complementary chains have reunited to a very limited extent is seen in the fact that all three markers retain some residual activity even though all the molecules have been denatured as judged by the optical density increase having reached its maximum. In a separate experiment, transforming DNA was heated at 120° in an autoclave for 10 minutes, cooled in air, and the streptomycin activity assayed: 0.05 per cent of the original activity remained. Since this very elevated temperature exceeded that required to denature even pure guanine-cytosine regions (110°),[4, 5] it is highly improbable that any DNA molecules escaped

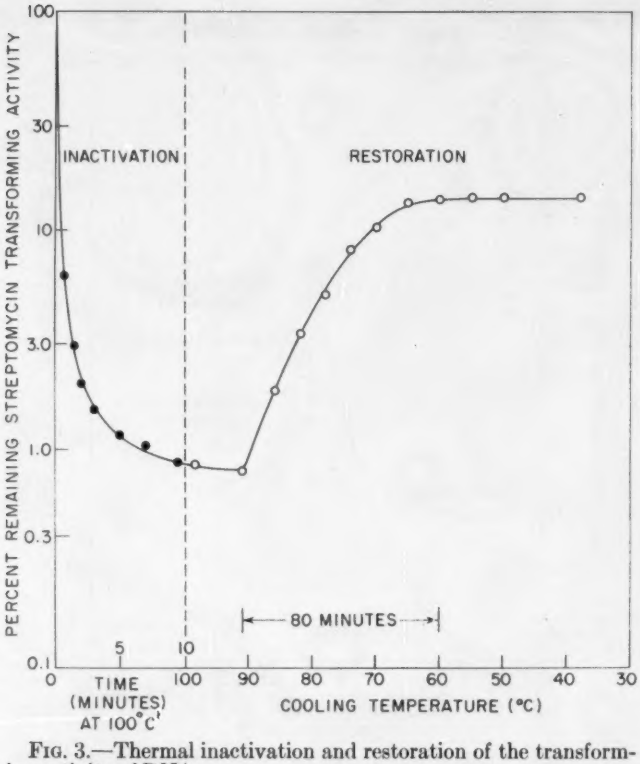

FIG. 3.—Thermal inactivation and restoration of the transforming activity of DNA.

Pneumococcal DNA at 20 μg/ml in standard saline-citrate was preheated for $1^1/_2$ minutes at 85.5° (no loss in biological activity), then transferred to a boiling water bath at 0 minute. At the times shown, samples were removed to an equal volume of 1.5 *M* NaCl plus 0.15 *M* Na citrate in an ice-water bath. After 10 minutes exposure at 100°, an equal volume of hot (100°) 1.5 *M* NaCl plus 0.15 *M* Na citrate was mixed with the DNA solution, the mixture transferred to a large water bath and then cooled slowly. During the gradual descent of the temperature, samples were removed (right of the dashed vertical line) to pre-chilled tubes in an ice-water bath.

denaturation. Thus the residual biological activity must be due to a low level of transformation by denatured DNA molecules or to a small amount of specific recombination to yield two stranded helices. The former possibility is not in accord with the finding that denatured DNA is not taken up by transformable cells.[13] Thus, specific reformation of the separated strands is indicated.

As a consequence a systematic investigation was undertaken of the variables that might affect the recombination so that what has been at best a marginal effect could be maximized. The important variables have been found to be the rate of cooling of the heated solution, the concentration of DNA and the ionic strength.

The effect of slow cooling: The transforming activity of thermally denatured pneumococcal DNA was found to be very dependent on the rate of cooling of the heated solution. Thus when pneumococcal DNA solutions are heated at 100° in standard saline-citrate for various periods of time and a series of aliquots removed, cooled quickly and slowly from 90° to room temperature, it is found that the slow

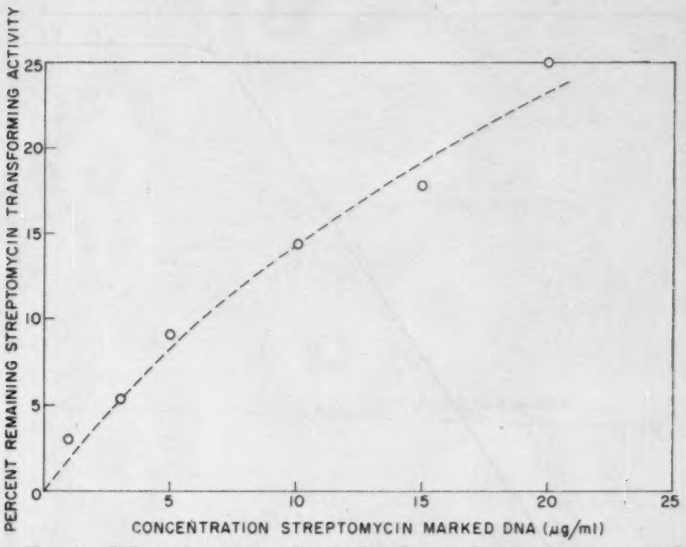

FIG. 4.—Effect of concentration during slow cooling on the recovery of transforming activity of thermally denatured DNA.

Pneumococcal DNA at 20 μg/ml in standard saline-citrate was heated for 10 minutes at 100°, transferred to a large bath at 89° and immediately diluted into pre-heated (89°C) tubes containing standard saline-citrate to give the final concentrations shown on the graph. After slow cooling all samples were diluted to the same concentration for assay. Circles are experimental points; dashed line is theoretical curve.[1]

cooling leads to an activity 35-fold greater than the fast cooling. This ratio is insensitive to the time of exposure at 100° between 5 and 20 minutes.

The transition from the activity characteristic of fast cooling to that characteristic of slow cooling can be seen in Figure 3. The transforming activity is plotted for quickly cooled aliquots taken from a solution during the exposure at 100° and then at various times during the slow cooling (at the right of the vertical dashed line) as the temperature of the bath dropped. Thus it is seen that restoration of the activity begins at about 90° and continues until about 65°. While the level of restored activity is about 15 per cent, this varies from sample to sample and in some cases has been found to be as high as 50 per cent. It is of interest to note that when fast cooled samples resulting in low biological activity are reheated and slowly cooled the transforming activity is restored to almost that of an aliquot that had been slow cooled originally. Thus the low activity of a fast cooled sample represents a repression rather than a destruction of its essential ability to transform. Essentially similar results have been obtained with thermally denatured transforming DNA isolated from *Streptococcus salivarius* and *Bacillus subtilis* on fast and slow cooling (Marmur, Lane, and Levine, unpublished results).

Effect of concentration of DNA during cooling: An important factor in determining the level of stored activity on slow cooling was found to be the concentration of the DNA itself during the cooling period. In contrast, there is practically no dependence of the activity on concentration for fast cooling. To illustrate the former a solution of pneumococcal DNA at a concentration of 20 μg/ml in standard saline-citrate was heated at 100° for 10 minutes and then diluted into hot solvent to give

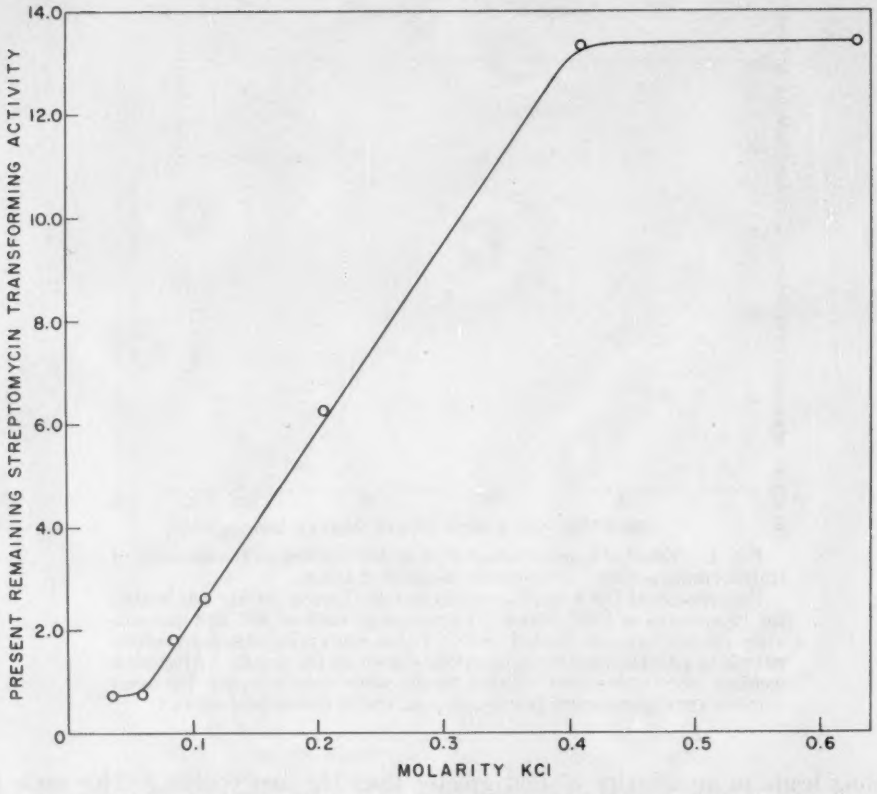

FIG. 5.—Effect of ionic strength on the recovery of transforming activity of slowly cooled denatured DNA.

Pneumococcal DNA at 200 μg/ml in standard saline-citrate was heated at 100° for 10 minutes, diluted 20-fold into KCl of various ionic strength and at 86°. The solutions were cooled slowly (the contribution of saline-citrate to the final ionic strength is included in the graph).

a series of concentration from 1 to 20 μg/ml. When these were slowly cooled the specific activities were found to increase nearly linearly from 3 per cent for 1 μg/ml to 25 per cent at 20 μg/ml (Fig. 4). As shown in the following article, this behavior is consistent with a bimolecular reaction.

Effect of salt concentration: Once the DNA strands have separated, the negatively charged phosphate groups would tend to prevent the reunion of the strands to form the helix. Increasing ionic strength would be expected to favor recombination by repressing this repulsive effect through shielding. This is shown to be the case in Figure 5. DNA was heated at a high concentration in standard saline-citrate at 100° for 10 minutes and then diluted into prewarmed tubes containing various concentrations of KCl. The solutions were cooled slowly, then assayed. There is a linear increase in biological activity between 0.05 *M* KCl and 0.4 *M* KCl. At higher concentrations of KCl (0.4 *M*) the biological activity reaches a maximum representing 14 per cent of the activity of the native material. Essentially similar results are obtained when the cooling is carried out in various concentrations of saline-citrate.

The effect of pH on strand reunion is broad with maximum at neutrality.

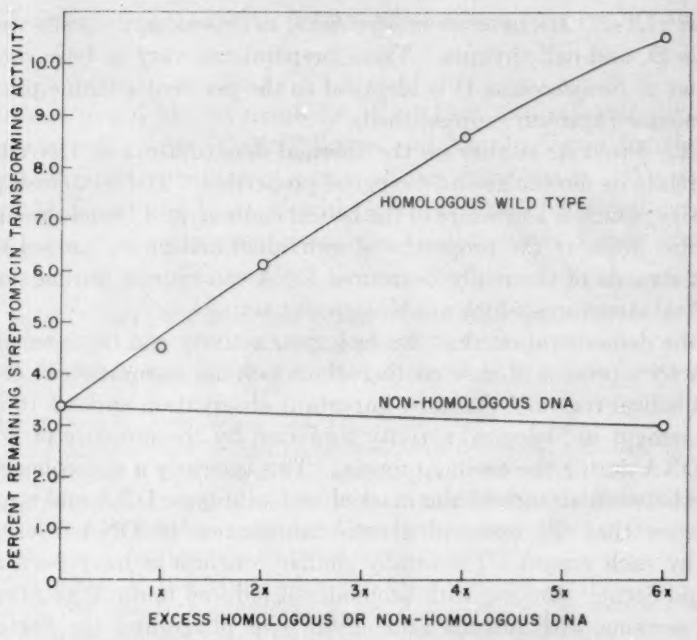

FIG. 6.—The effect of denatured homologous wild-type and heterologous DNA on the transforming activity.

Streptomycin marked pneumococcal DNA at 5 μg/ml was heated for 10 minutes at 97° in standard saline-citrate together with wild-type (homologous) pneumococcal DNA in the relative concentrations shown. Heterologous DNA and streptomycin marked DNA were also heated together. All the mixtures were slow cooled. Circles are experimental points; dashed line is theoretical curve.[1]

Effect of homologous and heterologous DNA: If the two complementary strands of the DNA molecule separate completely, it should be possible, by adding an excess of denatured homologous wild-type DNA during the slow cooling to reform hybrid molecules in which one strand carries a specific genetic marker and the other strand does not carry the marker. DNA isolated from streptomycin resistant pneumococci and various concentrations of DNA isolated from streptomycin sensitive cells were heated together in standard saline-citrate at 100° for 10 minutes and then cooled slowly. The resultant increase in biological activity is shown in Figure 6. Thus, it would appear that hybrid molecules formed in the slow cooling are active in carrying out transformations. Since transformants are selected and counted in a medium containing streptomycin, it is not known at the present time whether a cell transformed by a hybrid molecule results in a pure clone of streptomycin resistant cells or a mixed population of streptomycin sensitive and resistant cells. This question is now being studied using linked markers.

If genetically marked DNA is first denatured and then slow cooled in the presence of homologous DNA in the *native* state there is no increase of the biological activity over that of the control.

If during the slow cooling, denatured heterologous DNA is added at five to six times excess, there is again no increase in transforming activity over that of the control. The heterologous DNA samples tested have been isolated from *Salmonella*

typhimurium (LT-2), *Micrococcus lysodeikticus*, *Micrococcus pyogenes* var. *aureus*, *Streptococcus* D, and calf thymus. These preparations vary in base composition, although that of *Streptococcus* D is identical to the per cent guanine plus cytosine of *D. pneumoniae* (Marmur, unpublished).

Discussion.—Previous studies on the thermal denaturation of DNA have done little to correlate its molecular and biological properties. The relationship between optical density, which is a measure of the helical content, and transforming activity, which is a fine probe of the properties of individual molecules, has led to the discovery that strands of thermally denatured DNA can reunite during slow cooling to form helical structures which are biologically active.

Beyond the demonstration that the biological activity can be restored to denatured DNA by a process of slow cooling which permits restoration of complementary-paired helical regions,[1] the most important observation appears to be the additional increment in biological activity conferred by the presence of homologous wild-type DNA during the cooling process. This is clearly a consequence of forming hybrids between strands of the marked and wild-type DNA and strongly supports the view that the essential genetic information in DNA is carried independently by each strand. Essentially similar conclusions have been arrived at by Pratt and Stent[14] working with bromouracil induced mutants of phage and by Tessman[15] working with nitrous acid mutants of phage and the bacterial virus ϕX 174 which contains single-stranded DNA.[16] The duplicate set of genetic information in the native DNA molecule of the mutant strain is disseminated among a larger number of renatured molecules in which at least one strand carries the information. Closely related DNA from *Streptococcus* D (which can be transformed by pneumococcal DNA[17]) having the same over-all base composition as pneumococcus does not have the same effect as the added homologous denatured pneumococcal DNA during slow cooling. This points to the necessity for a close homology that is required for the interacting strands to form double stranded structures.

The question might arise as to how representative the biological results are of the whole molecular population. It has already been established that a genetic marker is associated with a DNA molecule,[18-20] occupying a small portion of it.[10] The number of DNA molecules in a pneumococcal cell is of the order of several hundred[18] if the weight average molecular weight is 10 million. Since essentially similar results have been obtained with both the streptomycin and bryamycin markers, and since they represent molecules which are relatively rich in guanine-cytosine and adenine-thymine respectively, it is safe to conclude that most of the molecules exhibit the same interactions as the molecules carrying the biological markers being assayed.

Summary.—DNA from *D. pneumoniae* when heated to temperatures where all the molecules are denatured still retains transforming activity. The activity is increased by slowly cooling the denatured DNA as well as by increasing the ionic strength and concentration of DNA during the cooling period. The reunion of the strands is specific and has been shown to occur between populations of denatured molecules of mutant strains of *D. pneumoniae*. The reunion does not occur between the DNA strands of pneumococcus and that from *Streptococcus* D or from organisms with widely varying base ratios. Hybrid molecules in which one strand contains the mutant marker and the other strand derives from wild-

type DNA are active in carrying out transformations with respect to the mutant property.

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STRAND SEPARATION AND SPECIFIC RECOMBINATION IN DEOXYRIBONUCLEIC ACIDS: PHYSICAL CHEMICAL STUDIES

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Communicated February 25, 1960

The separation and reunification of the complementary molecular strands of DNA, so clearly indicated by the restoration of biological activity,[1] can be demonstrated by physical chemical techniques as well. Such studies permit a more quantitative description of the phenomenon, make possible the inclusion of DNA samples that do not participate in bacterial transformation and lead to a better insight into the controlling features of the reaction. This, in turn, should provide a better basis for understanding the possibilities and limitations that DNA has

in vivo for the macromolecular reactions that are the counterpart of its genetic function.

In summarizing here our current work in this direction we begin by showing how strand separation and recombination is reflected in three physical chemical methods in the order in which we took them up. To proceed from these to more quantitative studies it became necessary to find reliable, routine means for determining the molecular weight of DNA in the various forms with which we were confronted, so that the complicating problems of aggregation and depolymerization could be assessed and minimized. Success along these lines made it possible to follow the molecular weight changes accompanying the processes being studied and to examine the effect of molecular weight thereon. In the final section we present the results of a study of recombination between strands which differ either in isotopic label or in species of origin. In both cases "hybrid" or "heterozygous" reformed molecules can be demonstrated.

OBSERVATION OF STRAND RECOMBINATION

Absorbance-Temperature Curves.—It has been established that when DNA solutions are slowly heated a dramatic macromolecular change occurs in a very restricted temperature range. The change is a cooperative melting out of the one-dimensional helical structure yielding disorganized, coiled polynucleotide chains. At 0.2 molar sodium ions the midpoint of this transition, T_m, lies in the interval of 80 to 100° depending on the guanine-cytosine content of the DNA.[2] The change, which involves a 40 per cent increase in absorbance, can be easily and accurately followed by measuring the absorbance at 260 mμ as a function of temperature.[3] When the solution is cooled to 25° the absorbance decreases until it is about 12 per cent above that of the original solution at room temperature. This appears to be due to the formation of short, imperfect, intrachain helical regions in which a major portion of the bases are paired. Upon reheating such solutions, in the case of calf thymus and other mammalian DNA, the absorbance increases gradually without a region of rapid rise since short, imperfect helices have a broad range of transition temperatures.[3]

When DNA of *Diplococcus pneumoniae* was thermally denatured and then reheated it was found, in contrast to the general behavior just described, that a distinct maximum occurred in the middle of the temperature range; beyond this there was a sharp rise coincident with the latter half of the original curve for native DNA. This behavior suggested that a typical helix-coil transition was taking place involving the denaturation of long, perfect helices containing about half of the bases in the DNA sample.

The meaning of this became more clear when the absorbance-temperature curves of quickly and slowly cooled samples were measured. Such results are shown in Figure 1. The rate of cooling of these samples is the same as that described in reference 1. The absorbance of the slowly cooled sample is seen to rise to a plateau and then continue to the abrupt region coincident with the thermal denaturation of the native DNA. This sample had retained a large degree of its original transforming activity as described in the foregoing paper.[1] From this it is concluded that the slowly cooled sample contained substantial amounts of the Watson–Crick helix. The curve for the quickly cooled sample, which had very low transforming

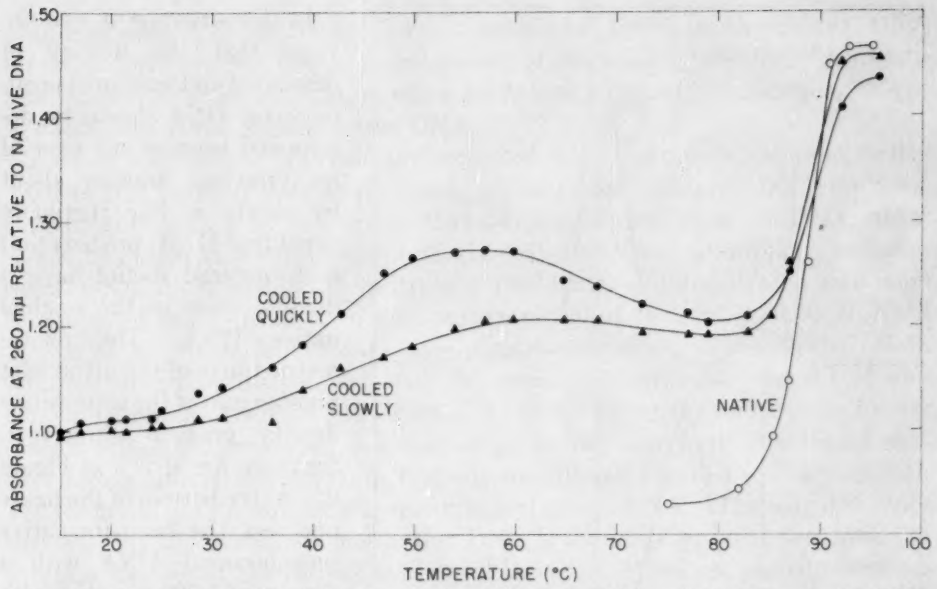

FIG. 1.—Thermal transitions of native, slowly cooled and quickly cooled *D. pneumoniae* DNA. *D. pneumoniae* DNA was heated at 100° for 10 min in standard saline-citrate. Hot concentrated saline-citrate was then added to a final concentration of 0.27 *M* NaCl plus 0.02 *M* Na citrate and subdivided into two portions, one cooled quickly in ice-water, the other cooled slowly. The native DNA was an aliquot of the same sample. The absorbance at 260 mμ, corrected for thermal expansion, was recorded after each solution was exposed for 15 min to the indicated temperature. The ordinate gives this reading relative to the absorbance of the native sample at 25°.

activity, can now be understood. At room temperature it is devoid of long, complementary helical regions, but during the gradual heating period short, imperfect helical regions melt out, releasing chains that then have time to develop long, complementary helices: these then melt in the vicinity of the characteristic temperature, T_m. Thus the display of the sharply melting region by the quickly cooled sample is seen to be an artifact in that this is not evidence that long, complementary helices exist in the same DNA sample at room temperature. This is consistent with its near absence of biological activity. We propose to call this form of DNA *denatured* and refer to the slowly cooled DNA with substantial amounts of reformed complementary helical regions as *renatured*.

Density Gradient Ultracentrifugation.—When a very dilute solution of DNA ($\sim$ 2 γ/ml) in about 7.8 molal cesium chloride is centrifuged at high speed in an analytical ultracentrifuge for 30–40 hours it is found that a density gradient has been set up and the DNA has migrated to a band at a position corresponding to its effective density. This is the basis of avaluable new technique[4] which permits the very accurate assessment of the density of DNA and, through the band profile, reflects in a combined fashion the molecular weight and the density heterogeneity of the DNA sample.[5] It has recently been shown that the density of DNA varies linearly with the guanine-cytosine content[6, 7] and that the distribution of guanine-cytosine among the molecules making up a DNA sample is relatively narrow, particularly for bacterial DNA.[6–8] It has been also shown that the density of DNA increases about 0.016 units upon denaturation.

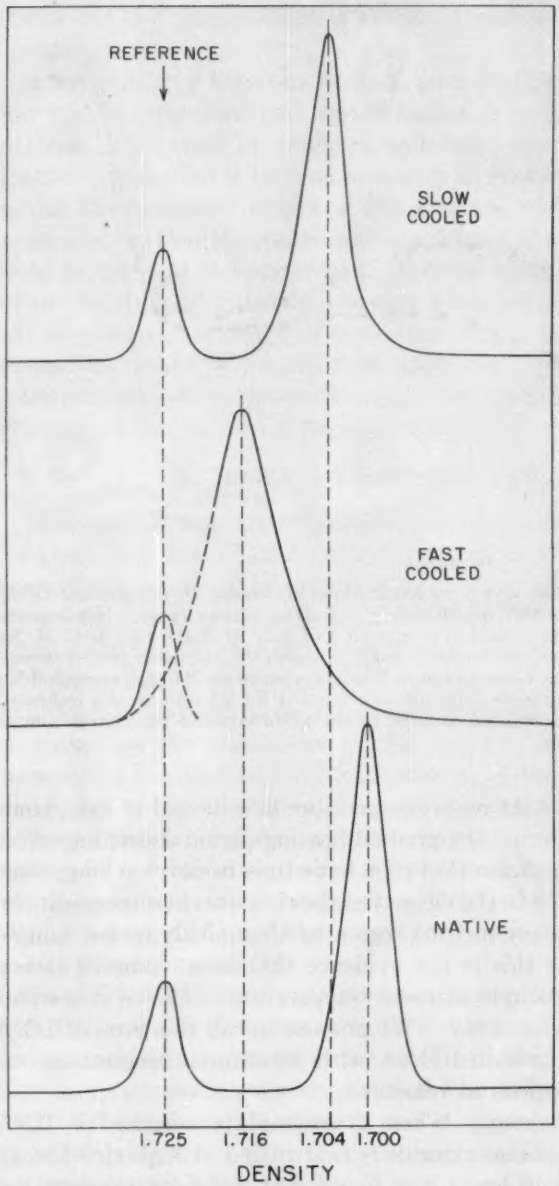

FIG. 2.—Molecular reconstitution in *D. pneumoniae* DNA.

Equilibrium concentration distributions of DNA samples banded in CsCl gradient. See text for a description of samples. Centrifugation at 44,770 rpm. The ordinate represents the DNA concentration as a function of the distance from the axis of rotation. Density increases towards the left. Conditions of heating and cooling are as described for Figure 1 of the preceding paper.[1]

In this situation it was obvious that the density of denatured and renatured pneumococcal DNA should be determined because our view of renaturation, brought about by slowly cooling thermally denatured DNA, predicts that such material should have a density close to the original native DNA. The photometric traces of the ultraviolet photographs of the appropriate density gradient ultracentrifugations are shown in Figure 2. At the bottom of the figure one sees the band for native pneumococcal DNA with a density of 1.700 together with a small band of DNA from *Pseudomonas aeruginosa* which is present in each case to provide a reference density. The trace for the quickly cooled, denatured pneumococcal DNA is shown in the middle exhibiting the expected higher density of 1.716. At the top is seen the tracing for the renatured DNA: it has a density of 1.704. Thus the renatured DNA is found to have a density close to that of native DNA supporting the view that it consists predominately of reformed, complementary helices of the same character, and density, as the native DNA.

Although the molecular weights of these three forms will be discussed at a later point it may be of interest to mention here that the pronounced widening of the band for the denatured DNA reflects an approximately five-fold reduction in molecular weight; hence only a part of this can be due to strand separation alone. The

near equivalence of the profile for the renatured DNA to the native DNA involves, therefore, not only a recombination of two strands but, under the conditions employed, some aggregation which by chance increases the molecular weight to about that of the original native DNA.

Electron Microscopy.—The evidence presented in the two previous sections clearly predicts that electron microscopy should reveal in the renatured DNA the long, cylindrical threads of 20 A. diameter that are characteristic of native DNA: these should be absent in the denatured (quickly cooled) DNA. Accordingly, pneumococcal DNA was heated in saline-citrate at 100° for 10 minutes, diluted with more concentrated saline-citrate to a final concentration of 18 γ/ml in 0.30 *M* NaCl + 0.03 *M* Na citrate, and cooled in two parts, one quickly and one slowly. Both solutions were then dialysed into 0.05 *M* ammonium carbonate plus 0.1 *M* ammonium acetate. Thereafter Professor C. E. Hall sprayed the solutions on freshly cleaved mica and, proceeding in a manner previously described[9, 10] obtained electron micrographs shown in Figure 3 (slowly cooled) and Figure 4 (quickly cooled). The former shows the structure characteristic of native DNA. The only difference between this and micrographs of native DNA is the more frequent occurrence of irregular patches at the ends of cylindrical threads. These are probably regions of denatured DNA arising from incomplete recombination or the inequality in length of the two strands that are paired. The micrograph for the quickly cooled sample (Fig. 4) shows only irregularly coiled molecules with clustered regions. These results are very similar to those found for ribonucleic acid.[11] Thus the identification of renatured DNA as being for the most part similar to native DNA and denatured DNA as being an irregular chain with considerable base pairing in short regions seems to be complete.

Dependence of Molecular Recombination on Concentration.—In the foregoing report[1] it is shown that the transforming activity of renatured pneumococcal DNA increases with the concentration at which it is slowly cooled (see Fig. 4 of ref. 1). This suggests that the extent of molecular recombination is quite low at concentrations considerably below those employed in the experiments described in the foregoing sections, that is, $\sim$ 20 γ/ml. In order to find out if the biological activity reflected the state of the DNA sample itself we heated and slowly cooled a pneumococcal DNA sample in the usual manner at a concentration of 1 γ/ml. The density of this sample was found to be 1.715. This value matches that of quickly cooled pneumococcal DNA (1.716) prepared either at the usual concentration, 20 γ/ml, or at this very low concentration, 1 γ/ml. Thus cooling at what we have described as a slow rate, that is from 90 to 60° in 80 minutes, does not lead to substantial recombination of strands when the concentration is quite low.

With the physical exhibition of the molecular recombination, as well as the biological, shown to be quite concentration dependent it seemed permissible to attempt to account for the concentration dependence observed (Fig. 4 of ref. 1) by assuming a bimolecular reaction, $A + A \leftrightarrows A_2$, which reaches effective equilibrium at a particular temperature that lies within the region where slow cooling takes place. If α represents the mole fraction of strands that are combined in the helical form, the equilibrium constant $K = \alpha/[(1 - \alpha)^2 c]$ where c represents the DNA concentration. This can be applied to the data in Figure 4 of reference 1 by

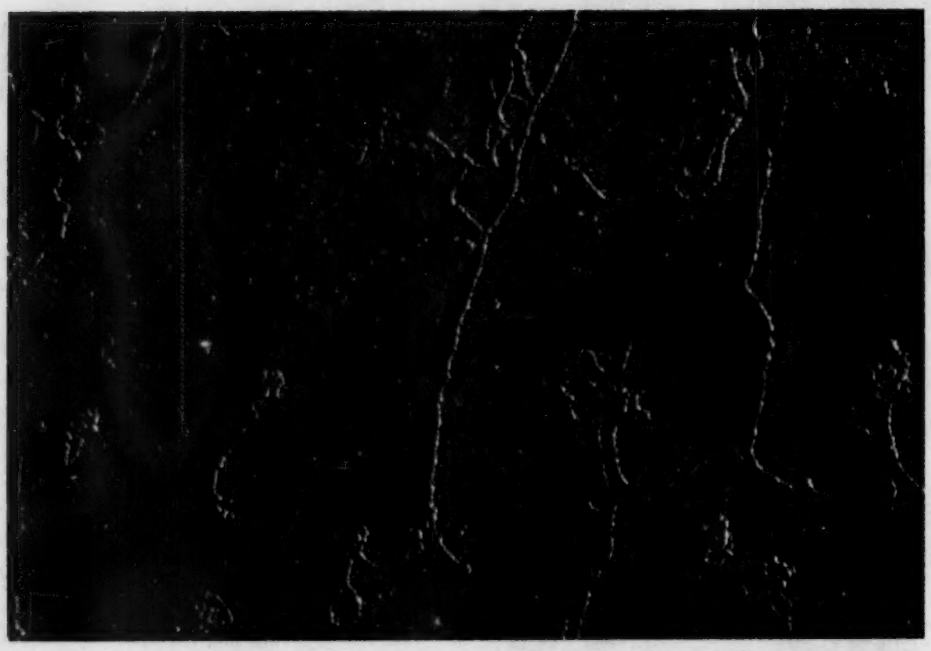

FIG. 3.—Electron micrograph of renatured *D. pneumoniae* DNA. Magnification 95,000 X. Shadow casting is from the right at a shadow to height ratio of 10:1. See text for heating and cooling conditions.

FIG. 4.—Electron micrograph of denatured *D. pneumoniae* DNA. Magnification 95,000 ×. The polystyrene reference sphere at the lower right of the micrograph has a diameter of 880 Å. Shadow casting is from the right at a shadow to height ratio of 10:1. See text for heating and cooling conditions.

assuming that α is equal to the fractional activity of the renatured DNA. By choosing one point to permit an evaluation of the product Kc, α can be calculated as a function of c. The dashed line drawn in Figure 4 of reference 1 is the result calculated in this way. Since it fits the experimental data there is reason to conclude that the molecular recombination is indeed bimolecular as the character of the Watson–Crick structure for DNA would demand. Moreover, the success of this simple approach suggests that properly designed experiments which yield K as a function of temperature should lead to the determination of the heat and entropy of the recombination as well as the free energy change involved in this process at the temperature of cellular division.

Dependence of Molecular Recombination on the Source of DNA.—With the view that molecular recombination in DNA consists simply of two complementary strands coming together with the fraction of strands complexed under control of an equilibrium constant it follows that the manifestation of molecular recombination depends critically on the concentration of complementary strands. Now the concentration of complementary strands is not identical with the concentration of DNA, when DNA from different sources is considered. Take the extreme cases, of typical bacterial DNA and the DNA of mammalian cells. Here the DNA content per cell differs by the order of a thousand fold. If we take the average molecular weight to be the same in both cases and if it is assumed that each DNA molecule in a given cell is different, it follows that at the same weight concentration, the concentration of complementary pairs will be a thousand fold greater for the bacterial than for the animal DNA. From this it follows that molecular recombination will not be expected for animal DNA under the conditions that are just sufficient to permit its occurrence in bacterial DNA.

The expectation that animal DNA will not show complementary reformation when bacterial DNA will do so has been borne out in two kinds of experiments.[12] In one set the absorbance temperature profiles for a number of denatured DNA samples have been determined. The majority of bacterial DNA samples showed some of the character seen in Figure 1, that is a sharply rising region at high temperature approximately coincident with the curve for native DNA. By contrast calf thymus and salmon sperm DNA showed essentially a continuous and gradual rise with temperature. This shows that on the time scale of the heating curve there was some molecular reconstitution in most of the bacterial samples but not in the cellular DNA.

In another experiment samples of thermally denatured DNA were cooled quickly and then brought to 80° and the absorbance followed as a function of time in 0.30 M NaCl plus 0.03 M sodium citrate. The pneumococcal DNA fell from 1.40 times the absorbance of the native DNA at 25° to 1.08 whereas the calf thymus showed no drop at all. This again demonstrates the inability of calf thymus DNA to reform the helical configuration under the conditions which do permit pneumococcal DNA to do so. Consequently, the great difference in concentration of complementary strands in the denatured DNA appears to offer the explanation.

THE MACROMOLECULAR PROPERTIES OF NATIVE, DENATURED, AND RENATURED DNA

A means of making reasonably accurate molecular weight assignments to DNA in each of the forms under study is necessary in order to examine quantitatively

the phenomenon of molecular recombination in DNA and to enable the complicating features of aggregation and depolymerization to be understood and eliminated in so far as possible. Although light scattering measurements were the first method to satisfactorily establish the molecular weight and shape of DNA in solution,[13, 14] its accuracy and applicability in routine use, particularly for molecular weights greater than 5 million, has been found wanting elsewhere[15] as well as in this Laboratory. Consequently, a systematic investigation of sedimentation constants, evaluated by extrapolation to zero concentration, and intrinsic viscosities evaluated by extrapolation to zero gradients, has been undertaken[16] and molecular weights have been derived by a careful application of the Mandelkern–Flory equation:[17]

$$M = \left[\frac{s^\circ [\eta]^{1/2}\eta_0 N}{\beta(1 - \bar{v}\rho)}\right]^{3/2}$$

In this equation, s° = the sedimentation constant at zero concentration, $[\eta]$ = the intrinsic viscosity at zero gradient, η_0 = the solvent viscosity, $N = 6.03 \times 10^{23}$, $(1 - \bar{v}\rho)$ = the buoyancy factor and β = a constant which has a value which ranges from 2×10^6 to about 4×10^6 as the permeability of a coiled molecule or the axial ratio of a rigid ellipsoid increase. For most flexible polymers the value of β has been found to be about 2.6×10^6.

Native DNA.—An investigation[18] of DNA samples in which the molecular weight had been varied by ultrasonic radiation and the molecular weights measured by light scattering showed β to increase monotonically from 2.56 at 300,000 to 3.29 at 8,000,000. Other light scattering experiments, and an independent study[19, 20] in which molecular weights were determined by sedimentation-diffusion, have shown good agreement with this result. Such an empirical evaluation of β, based as it is on absolute methods, permits us to use the Mandelkern–Flory equation to derive weight average molecular weights from sedimentation-viscosity data.

TABLE 1

VALUES OF s° AND $[\eta]$ FOR SELECTED MOLECULAR WEIGHTS OF NATIVE DNA

Molecular Weight	$s^\circ_{20,w}$ in HMP	$[\eta]$ in HMP	$s^\circ_{20,w}$ in SSC	$[\eta]$ in SSC
1,000,000	8.8 *S*	9.1 dl/gm	10.0 *S*	8.3 dl/gm
2,000,000	11.5	21.0	−13.1	18.7
4,000,000	15.4	45.5	17.5	38.0
10,000,000	22.9	120	26.4	82.5
16,000,000	28.2	190	32.8	119

For a number of samples s° and $[\eta]$ have been measured in a solvent of lower ionic strength, 0.011 *M* in Na^+ ions, as well as in the more common solvent that is 0.195 *M* in Na^+ ions. The former solvent contains 0.0025 *M* Na_2HPO_4, 0.0050 *M* NaH_2PO_4 and 0.001 *M* sodium ethylenediaminetetraacetate and has a pH of 6.8. For convenience this is referred to as HMP (hundredth molar phosphate). The more common solvent consists of 0.15 *M* NaCl and 0.015 *M* $Na_3(C_6H_5O_7)$ with a pH of about 7. This will be referred to as SSC (standard saline-citrate). On a double logarithmic plot of s° against *M* the data in these two solvents fall on two parallel lines exhibiting slight upward curvature. Similarly, log $[\eta]$ against log *M* yields lines with downward curvature. Table 1 summarizes the smoothed $s^\circ_{20,w}$ and $[\eta]$ values in these two solvents for selected molecular weights.

Denatured DNA and the Problem of Aggregation.—When DNA is thermally denatured and cooled, a large fraction of the bases become paired.[3] While most of this pair formation can occur within each chain, some interchain bonding can be expected and this would increase with DNA concentration and molecular weight. Consequently, the determination of the molecular weight of denatured DNA requires proof that aggregation has been eliminated. Early light scattering studies showed no significant change in molecular weight on thermal denaturation,[21] and thereby suggested that the two strands had not separated. Other reports claimed a decrease to one-half the molecular weight but they were not convincing.[22] Moreover, the s° and $[\eta]$ values for such denatured DNA did not yield a molecular weight in agreement with the light scattering values by a wide margin. Upon further investigation of this dilemma we have been able to show that significant aggregation occurs upon cooling denatured DNA in SSC at concentrations in excess of 40–100 γ/ml. Since light scattering and viscosity measurements require concentrations in this range or higher we were able to conclude that aggregation had occurred in most samples previously studied.

In order to eliminate this aggregation in the useful range of concentration we have gone to a solvent of lower ionic strength, HMP. Although there is some reformation of base pairs (about 25 per cent) in this solvent at room temperature no aggregation is evident by any of the methods employed and a satisfactory dependence of s° and $[\eta]$ on molecular weight has been established by using the Mandelkern-Flory equation with a value of 2.6×10^6 for β. The results of such studies can be fitted with the usual empirical relations: $s^\circ = K_s M^{a_s}$ and $[\eta] = KM^a$.

Now it has been shown that the guanine-cytosine bond is stronger than the adenine-thymine bond[2, 8] and as a result it is to be expected that the amount of base pairs in denatured DNA will increase with the guanine content. Indeed this has been shown to be the case with RNA samples of different composition.[3] Consequently it is possible that the dependence of s° and $[\eta]$ on molecular weight for denatured DNA will depend on the composition of the DNA. This has been found to be the case and it is illustrated in Table 2 where the constants for the

TABLE 2

CONSTANTS FOR THE EMPIRICAL SEDIMENTATION AND VISCOSITY RELATIONS FOR DENATURED DNA IN PHOSPHATE-VERSENE SOLUTION 0.011 M IN Na^+ (HMP)

Source of DNA	K_s	a_s	K	a
D. pneumoniae	0.054_5	0.35_4	$3.4_6 \times 10^{-5}$	0.93_3
E. coli K-12	0.055_8	0.36_1	$3.1_1 \times 10^{-5}$	0.91_2

empirical equations are listed for denatured DNA (quickly cooled) from *D. pneumoniae* and *Escherichia coli* (K-12).

These results indicate a rather highly swollen chain configuration for DNA, despite some base pair interaction. For a given chain length the molecular dimensions that can be deduced from this are in the range expected for typical polyelectrolytes. These dimensions are much larger than those for RNA of the same chain length and in the same solvent: this is probably due to the additional contraction of the RNA coil due to additional hydrogen bonding made possible by the 2-OH of the ribose ring.

With these results at hand it is possible to assess the molecular weight as a function of time of heating at a given temperature and to demonstrate whether or not strand separation has occurred at the early stages of heating.

Strand Separation and Depolymerization.—In the low ionic strength solvent (HMP) the midpoint of the thermal denaturation profile, T_m, for *D. pneumoniae* is shifted downward from 85° in SSC to 64° and the transition is complete at 70°. For *E. coli* DNA the corresponding values are 5° higher. A temperature 15° above the T_m is well beyond the melting out region for DNA in this solvent, indeed by about the same amount as 100° is for the higher ionic strength solvent (SSC). Consequently 79° and 84° were chosen as the temperatures at which to follow the thermal degradation of these two DNA samples. Aliquots were removed periodically from a large amount of the solution that had been brought to

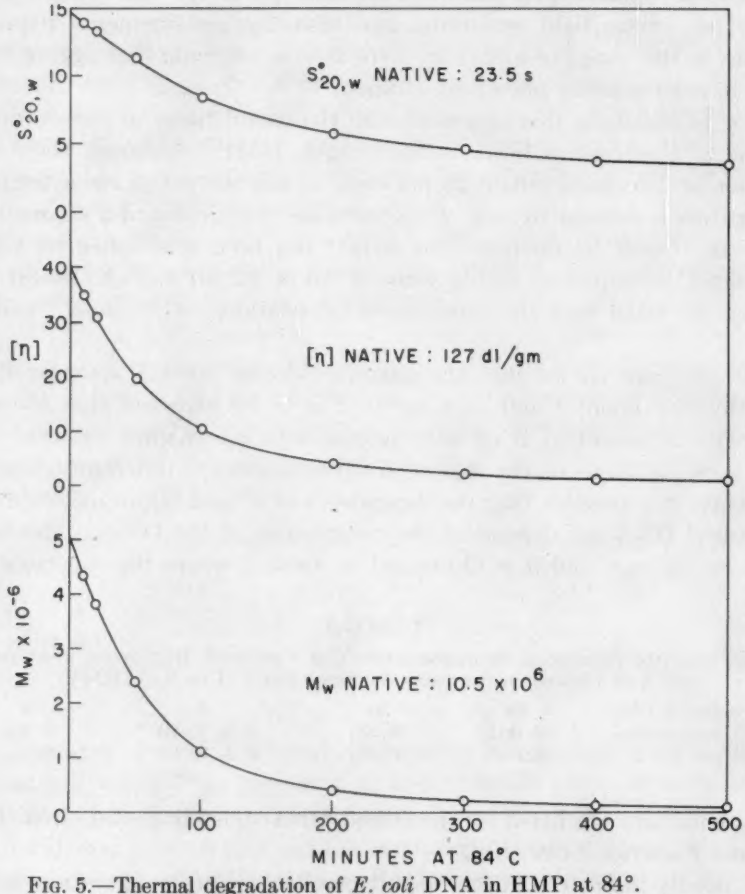

FIG. 5.—Thermal degradation of *E. coli* DNA in HMP at 84°.
Molecular weights derived from Mandelkern-Flory equation[17] using $\beta = 2.6 \times 10^6$.

this temperature, and cooled quickly. The s° and $[\eta]$ were measured at 25° and the molecular weight calculated as indicated above. The results for *E. coli* DNA are shown in Figure 5. It is seen that the molecular weight falls continuously but rather slowly through the period of exposure to elevated temperature. The ex-

trapolation back to zero time is obvious and the molecular weight value there is 5.0 million. The molecular weight of the native DNA was 10.5 million ($s^{\circ}_{20,w}$ = 23.5 S and $[\eta]$ = 126). In this case we have, therefore, a clear example of the molecular weight decreasing by a factor of two upon thermal denaturation, and with aggregation eliminated and depolymerization taken into account it can be said that strand separation did occur in the very early stages of the exposure to the elevated temperature.

The behavior of pneumococcal DNA in a similar experiment was roughly the same except that in this case the molecular weight fell from 8.2 million for the native DNA to 2.9 million for the denatured DNA at zero time. This change by a factor of somewhat more than 2, in this case 2.83, probably reflects an occasional enzymatically induced single chain scission in the original DNA preparation.

From the data obtained during the first 100 minutes of the above experiments the rate of thermal depolymerization of the polynucleotide chains of DNA can be evaluated. In practical units this can be expressed as 0.165 scission per 10 million molecular weight per 10 minutes at 79°. Similar experiments at 100° yields 1.41 in the same units. These and other results lead to a value of about 20 kcal for the activation energy for hydrolytic scission of individual strands of DNA, a value similar to that found in a quite different study of ribonucleic acid.[23] With this evaluation of the rate of bond scission it is possible to assess the extent of fragmentation induced by the exposure to 100° for 10 minutes followed by fast or slow cooling. For fast cooling the value stated above is the answer desired since the units have been chosen for convenience in this situation. Since pneumococcal DNA is often in the range of 10 million molecular weight each native molecule will receive on the average between one and two scissions as a result of the heating to 100°. Slow cooling will involve a longer exposure to temperatures where degradation is significant: in the case of our particular cooling rate the depolymerization would be about doubled.

Renatured DNA.—Renatured DNA was prepared by heating pneumococcal DNA of molecular weight 8.2 million at 100° for 10 minutes in standard saline-citrate at a concentration of 20 γ/ml and cooling slowly in double the saline-citrate concentration. In standard saline-citrate this material had a $S^{\circ}_{20,w}$ of 23.5 S and an $[\eta]$ of 21.6 from which a molecular weight of 6.0 million was deduced. An aliquot of the same heated solution, quickly cooled, had a molecular weight of 2.0 million. When dialyzed into the lower ionic strength solvent (HMP), the renatured DNA had a $s^{\circ}_{20,w}$ of 15.4 S and an $[\eta]$ of 43. A molecular weight of 4.0 million was obtained using these results. The denatured DNA showed no change in molecular weight when similarly transferred to the lower ionic strength solvent.

From these results it appears that a small amount of aggregation occurs when renaturation is carried out at 20 γ/ml in 0.3 M NaCl plus 0.03 M Na citrate and that the molecular weight falls by about the amount expected from the depolymerization rates reported above. In the lower ionic strength solvent, where the aggregation appears to have been eliminated, it is interesting to note that the s° and $[\eta]$ values are essentially equal to those given in Table 1 for 4 million molecular weight native DNA. This supplies further evidence of the close structural similarity of the renatured to the native DNA.

The results have an important bearing on the transforming activity that can be

expected for renatured pneumococcal DNA. The loss in activity to be expected from the molecular weight reduction can be obtained directly from an earlier study[24] of the molecular weight dependence of transforming activity. For a reduction of 10 to 4 million the activity should fall to 70 per cent of its original value. However, the activity would be expected to be lowered still further due to the fact that the complementary strands that recombine will generally be of different length as a result of the mild thermal depolymerization. If there was no selection according to chain length in recombination there would be a loss of about 50 per cent of the nucleotides from helical regions due to this effect and a drop to 35 per cent would be expected for the maximum activity. However, there is probably some preference for complementary chains of more nearly equal molecular weight to recombine and the evidence from the absorbance-temperature profile and DNA density in CsCl indicates that about 75 per cent of the nucleotides do reform in the helical configuration. Consequently the maximum biological activity of renatured transforming DNA under the conditions employed may be placed at about 50 per cent. The highest observed[1] has been approximately 50 per cent.

RECOMBINATION OF DNA STRANDS OF DIFFERENT DENSITY AND FROM DIFFERENT SPECIES

In this final section we return to the technique of density gradient ultracentrifugation and ask if by its use recombination between strands of different density can be observed. Two cases are of interest. In one we mix two DNA samples from the same species, one of which is denser by virtue of having N^{15} instead of N^{14}. In the other we mix N^{15} *E. coli* DNA with N^{14} DNA from other bacteria to see if renatured molecules of intermediate density can be observed. Such "heterozygous" molecules may be expected from closely related bacteria but not from distantly related ones.

From what has been stated in the previous section concerning aggregation and strands of unequal length in the renatured molecules, it is not surprising to find that the optimal resolution of hybrid molecules has proved somewhat elusive. Thus, while maximum helix development in the renatured sample is favored by high DNA concentrations, so is aggregation. Obviously aggregation of renatured molecules of different density will smear out the hybrid band one seeks to resolve. Likewise the inequality of chain lengths in the hybrid molecules will spread out their densities with a corresponding broadening of the hybrid band.

Against this background we present the results obtained with a mixture of N^{14} and N^{15} *E. coli* DNA each at a concentration of 5 γ/ml. This concentration is low enough to prevent aggregation but at the price of only limited recombination to the helical form. The results are collected in Figure 6. At the top (*A*) is shown the tracing for the quickly cooled, denatured DNA. The bands are seen to be completely separated without any hybrid formation. Thus, the reforming of base pairs has been entirely intrachain.

If an aliquot of the same heated mixture is slowly cooled the results are as shown in the second tracing (*B*). Here the peaks have remained at the same densities as in the denatured DNA but about one-third of the total DNA has shifted to a lighter density distributed about the value of 1.724. While this is indicative of hybrid formation it is far from proof since the densities of the peaks are essentially

those of denatured N^{14} and N^{15} DNA and do not approach that of renatured material which should be about 0.008–0.012 density units lighter than the denatured materials.

In this situation it is necessary to provide a control consisting of the mixture of the two separately cooled DNA samples, that is, samples that have had the same thermal history as the slowly cooled mixture (*B*) but have been prevented from forming hybrids. The tracing of this control is shown in the Figure as (*C*). This provides a key to our interpretation since peaks corresponding to the renatured molecules are clearly evident at the right of each of the denatured peaks.

If the control *C* is now subtracted from the trace for the slowly cooled mixture (*B*) the migration of material to the density corresponding to renatured hybrids should be evident. This is shown at the bottom as (*D*). A shift of material from extremities to the density of the hybrid is clearly evident. About 20 per cent of the total DNA is involved: this is about that expected from (*C*) since there it can be seen that about 40 per cent of the material is in the bands corresponding to the renatured form.

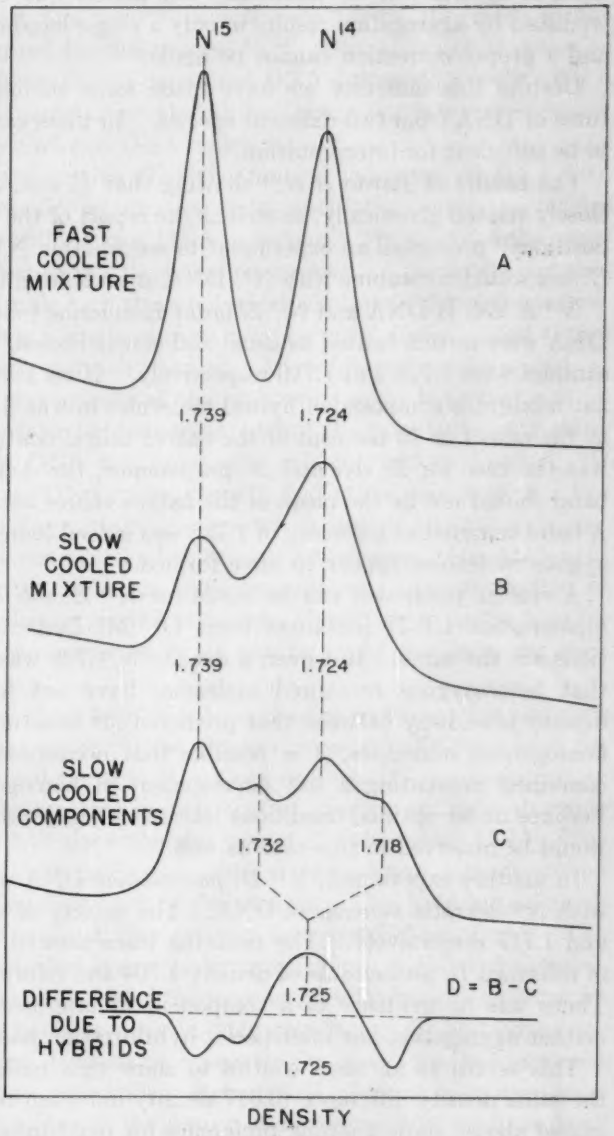

FIG. 6.—Hybrid formation in *E. coli* B DNA. Equilibrium concentration distributions of DNA samples banded in CsCl gradient. See text for a description of samples. Centrifugation at 44,770 rpm. The ordinate represents the concentration of DNA in the centrifuge cell. The area of each band is proportional to the amount of DNA it contains. N^{15} and N^{14} labeled *E. coli* B DNA were prepared by growing wild-type cells in a synthetic medium containing $N^{15}H_4Cl$ or $N^{14}H_4Cl$, respectively, as the sole nitrogen source. The DNA was isolated by a procedure to be published (J. Marmur, in preparation).

By using 4 times the concentrations employed in the above experiment the de-

natured species can be essentially eliminated but the additional spreading introduced by aggregation results in only a single band of somewhat irregular shape, and a proper correction cannot be made.

Despite this difficulty we have made some preliminary observations on mixtures of DNA from two different species. In these cases the peak densities appear to be sufficient for interpretation.

The results of Baron *et al.*[25] showing that *E. coli*, *Shigella* and *Salmonella* are closely related genetically, as well as the report of the similarity of their base compositions[26] prompted an experiment to see whether N^{15} labeled DNA strands from *E. coli* would recombine with N^{14} DNA strands from the other enteric organisms.

N^{15} *E. coli* B DNA and N^{14} *Shigella dysenteriae* (obtained from Dr. S. E. Luria) DNA were mixed, heated as usual and slowly cooled. The densities of the native samples were 1.725 and 1.710 respectively. If we assume 50 per cent of the resultant mixture is composed of hybrid molecules in which helical regions have formed to the extent of 75 per cent of the native helical content we would expect that, as was the case for *E. coli* and *D. pneumoniae*, the density of the resultant hybrid band should not be the mean of the native values but 0.004 density units heavier. A band centered at a density of 1.722 was indeed found and thus hybrid, or heterozygous molecules appear to have formed.

A similar prediction can be made for N^{15} *E. coli* B DNA and N^{14} *Salmonella typhimurium*(LT-2) (obtained from Dr. M. Demerec) since the base compositions are the same. However, a density of 1.729 was observed. Thus it appears that heterozygous renatured molecules have not formed. Since the observed density is midway between that predicted for denatured molecules and renatured, homogenous molecules, it is possible that occasional heterozygous regions have combined preventing a full development of homogeneous renatured molecules. Perhaps under optimal conditions interspecies molecular hybridization of the DNA would be observed in this case as well.

In another experiment, N^{14} *D. pneumoniae* DNA was heated and slowly cooled with N^{14} *Serratia marcescens* DNA. The density of the native samples are 1.700 and 1.717 respectively. The resulting trace showed two clearly separated bands of reformed *D. pneumoniae* of density 1.704 and reformed *Serratia* of density 1.721. There was no evidence of a component of intermediate density indicating that neither aggregation nor interspecies hybridization had occurred.

This serves as an ideal control to show that molecules having approximately the same density difference (0.017 density unit) but not the genetic similarity discussed above, show a strong preference for renaturing only with members of their homologous species. Only in the case where similarity in base composition has been correlated with genetic interaction has it been possible to demonstrate the appearance of heterozygous molecules.

Discussion.—Our aim in this paper has been to present a series of preliminary reports of closely related work on strand separation and molecular recombination in DNA. Consequently, the discussion will be postponed until the fuller accounts are published. Nevertheless, perhaps two points may be emphasized briefly.

The demonstration that strand separation actually occurs in a matter of a few minutes or less eliminates some of the objections[27] pertaining to the unwinding of DNA that have been put forward as a criticism of the Watson–Crick mechanism[28]

for DNA duplication. What has been observed here favors the more recent calculations of the time required for this process.[29, 30] Also, although it is not as yet apparent whether the reformation of denatured DNA during slow cooling has a biological counterpart, the demonstration that it does occur suggests a mechanism for genetic exchange in closely related DNA molecules.

The second point is the recognition that the routine reduction of DNA into single strands and their specific reformation into essentially native molecules provides a means of creating entirely new DNA molecules. The study of the progeny of such heterozygous molecules will be of obvious interest. A particular instance is that of a heterozygous molecule each strand of which contains a different marker. The progeny from cells having a single nucleus may be pure clones, mixed clones or transformants with markers normally linked. Any of these results would, of course, illuminate the molecular aspects of the replication act. If linkage can be found, not only will crossing-over be demonstrated but the possibility of making *in vitro* new forms of viable DNA not previously existent will be assured. It seems likely that heterozygous DNA molecules will find other uses as well. For example, active hybrid molecules containing a greater variety of alterations in one strand. New possibilities of testing whether or not homologous ribonucleic acid has sequences in common with DNA can now be explored. The formation of molecular hybrids between closely related organisms should be a useful tool for plotting homologies in base sequences where no genetic exchanges have yet been demonstrated.

Summary.—When solutions of bacterial DNA are denatured by heating and then cooled, two different molecular states can be obtained in essentially pure form depending on the choice of conditions, that is, rate of cooling, DNA concentration, and ionic strength. One state corresponding to fast cooling consists of single stranded DNA having about half the molecular weight of the original DNA. The other state corresponding to slow cooling consists of recombined strands united by complementary base pairing over most of their length. This form has as much as 50 per cent of its original transforming activity and is called renatured. The quickly cooled, single stranded form is essentially inactive and is called denatured. These two forms are clearly identified by differences in (1) absorbance-temperature curves, (2) density, (3) appearance in electron micrographs and (4) hydrodynamic properties. The recombination depends on concentration in the manner expected for an equilibrium between two independent strands and a bimolecular complex.

Molecular weight determinations of the native and renatured forms have been based on an extension of an earlier calibration of sedimentation and intrinsic viscosity in terms of light scattering measurements. For the denatured form molecular weights were determined by the use of the Mandelkern-Flory relation. By these means thermal degradation was accurately assessed. To avoid aggregation it was necessary to substantially lower the cation concentration over that previously used. In this way denatured DNA has been characterized as a single-chain, unaggregated polymer and strand separation demonstrated.

Density gradient experiments on N^{14} and N^{15} *E. coli* DNA have shown the the existence of hybrids in the DNA renatured from the mixture. Similarly, hybrids have been shown to form between the strands of bacteria that are closely related genetically. Thus the possibility of forming by renaturation heterozygous DNA

molecules with different genetic markers or chemical modifications in the two strands seems assured.

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THE RELATION OF UREA TO THE MOVEMENT OF WATER IN LIVER TISSUE

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The purpose of this study has been to determine what conditions maintain the high level of osmotic pressure within liver cells present immediately after their removal from the body, to define the relation of water movement to this pressure, and to learn if intracellular osmotic pressure and water movement are changed when amino acids and related substances are transformed to produce urea.

Significant information concerning the activity of liver or of kidney cells as osmometers is obtainable immediately after the tissue is removed from the body.[1] With relation to a wide variety of electrolytes, liver cells acting as osmometers measure the osmotic pressure of solutions in accord with the molecular weight, valence, and ion-dissociation of each substance. Tables (Robinson and Stokes[2]) assembled from freezing point depression, vapor tension, electrical conductivity, and other colligative properties of electrolytes have made it possible to define the molar concentration of electrolyte solutions with the same osmotic pressure. A solution of sodium chloride isotonic for liver immediately after its removal from the body is approximately 0.34 molar and a similar solution for kidney, 0.23 molar. Water equilibrium is maintained by the tissue at these levels during 15 to 20 min. Electrolytes as varied as potassium chloride, with monovalent base, magnesium chloride, with bivalent base, and lanthanum chloride with trivalent base, are isotonic with liver or kidney tissue in solutions that have the same osmotic pressure as that of sodium chloride in solution isotonic with one or other of these tissues. The tissues, acting as osmometers, measure osmotic pressure with approximate accuracy.

The plasma membrane of liver cells is evidently imperfectly semipermeable to solutions of electrolytes and with their entrance into the cell permeability to both electrolyte and water is increased. Maintenance of isotonicity *in vitro* is promoted by immersion in media with electrolytes similar to those of blood plasma, by temperature at 38° C and by an adequate supply of oxygen.[5]

Slices of liver tissue, weighing preferably from 60 to 90 mg, determined rapidly by means of a torsion balance, have been immersed in the Krebs[3] modification of Ringer's solution much used for manometric measurement of oxygen consumption. The solution with electrolyte concentration of 0.154 molar has been buffered with potassium phosphate and treated with oxygen or buffered with sodium bicarbonate and gassed with oxygen, 95 per cent, and carbon dioxide, 5 per cent. The medium is hypotonic for liver tissue but when its molar concentration is increased two-fold by addition of sodium chloride isotonicity may be maintained during one or two hr.[4]

Water movement measured in per cent of weight before immersion varies widely with oxygen supply to the medium. With abundant oxygen under conditions that promote its diffusion throughout the medium and into the tissue, water enters rapidly during about one hr but later is lost and within 2 or 3 hr may be less than that before immersion. With anoxia caused by diminished oxygen supply or by substitution of nitrogen for oxygen, water intake during the first hr of immersion

may not be significantly different from that with oxygen, but later water of the tissue increases during the next two or three hr.[5]

The opinion that the osmotic activity of tissues is determined by their electrolyte content, chiefly by potassium and sodium, has had wide acceptance but there is much evidence to indicate that protein and substances concerned with metabolism may have an important part in maintaining intracellular osmotic pressure. When liver tissue is subjected to a temperature approximately 38°, it undergoes autolysis and if slices prepared from tissue that has been kept at this temperature are immersed in solutions of sodium chloride with concentration graded from 0.1 to 0.5 molar, the level of isotonicity will be found to have increased with the duration of incubation (Table 1) so that after one and a half hr it may have reached 0.8 molar. This change is best explained by the assumption that autolysis, with disintegration of protein, has resulted in the formation of organic substances with relatively small molecular weights. When liver slices are made from tissue that has been kept at approximately 0°C, its level of isotonicity does not increase (Table 2).

TABLE 1

ISOTONICITY OF LIVER TISSUE INCUBATED AT 38°C

	Isotonic with solutions of sodium chloride molar
Before immersion	0.37
After 30 min	0.48
After 60 min	0.51
After 90 min	0.81

Small blocks of liver tissue, all from the same animal, have been incubated at 38°C for periods varying from 30 to 90 min. Slices prepared from them have been immersed in graded solutions of sodium chloride during 10 min.

TABLE 2

ISOTONICITY OF LIVER TISSUE KEPT AT 0.3°C

Experiment	Isotonicity of liver tissue molar	At 0.3° min	Isotonicity of liver tissue kept at 0.3°C molar
1	0.37	120	0.38
2	0.36	125	0.39
3	0.38	145	0.35
4	0.27	153	0.20
Av	0.35	...	0.34

Small blocks of liver tissue have been kept in broken ice and water (approximately 0.3°C) during the time indicated. Slices have been prepared and immersed in graded solutions of sodium chloride, 0.1 to 0.5 molar, during 10 min.

The molar concentration of solutions of a wide variety of electrolytes isotonic with liver tissue coincides with the osmotic pressure each maintains because the liver cell is an approximately exact osmometer,[1] but similar information concerning organic nitrogenous substances is not readily available. Nevertheless, it is possible to determine for some nitrogenous substances the molar concentration that is isotonic with liver and to compare it with the concentration of sodium chloride similarly isotonic with liver tissue immediately after its removal from the body (Table 3).

Wide variation in the osmotic pressure maintained by amino acids, urea, and some nitrogenous substances related to them suggest the possibility that these substances, intimately associated with protein metabolism, may have a part in determining intracellular osmotic pressure and movement of water.

The formation of urea by the action of arginase on arginine was described by Kossel and Dakin.[6] The reactions by which arginine, ornithine, and citrulline form

urea were defined by Krebs and Henseleit.[3] These substances (Table 2) are isotonic with liver cells in molar concentration approximately that of sodium chloride, whereas urea and closely related substances which are readily diffusible are isotonic with liver cells only when in highly concentrated solution. It has seemed probable that protein, amino acids, especially arginine, together with ornithine and citrulline and doubtless other nitrogenous substances, contribute to the osmotic pressure within liver cells. This relation suggests that intracellular pressure is perhaps diminished when urea is formed, and experiments have been made to determine if water movement in liver slices is related to the formation of urea.

TABLE 3

MOLAR CONCENTRATION OF SOLUTIONS OF UREA, AMINO ACIDS, AND RELATED SUBSTANCES ISOTONIC WITH LIVER TISSUE

	Molar
Sodium chloride	0.34
Arginine	0.26 (3)
Arginine hydrochloride neutralized	0.24 (1)
Ornithine hydrochloride neutralized	0.25 (2)
Guanidine hydrochloride neutralized	0.29 (3)
Choline chloride	0.28 (2)
Citrulline	0.47 (2)
Creatinine	0.49 (2)
Glycine	0.72 (1)
Alanine	0.71 (2)
Thiourea	0.85 (2)
Methyl urea	1.26 (2)
Urea	1.56 (3)

The number of determinations is shown by the figures in parenthesis. The hydrochlorides of arginine, ornithine, and guanidine have been neutralized with choline hydroxide (pH 7).

The colorimetric method of Archibald[7] has been used to measure urea formation. Protein has been removed from homogenates of tissue slices and from immersion media by heating to 100°C, in the presence of a sodium acetate buffer. Urea is determined by the red color which appears when it is heated with alpha-isonitrosopropiophenone.

Several procedures have been tested to determine *in vitro* the relation of urea formation to oxygenation of the medium. Liver slices have been immersed in Krebs-Ringer solution buffered with potassium phosphate and supplied with oxygen (100 per cent) allowed to enter at the bottom of a large test tube (low bubbling) or about 0.5 cm below the surface of the column of fluid in the tube (high bubbling). With the less effective oxygenation of the high bubbling gas there has been a slight increase of urea above that in the tissue before immersion, whereas with oxygen bubbling into the lowermost part of the fluid and in contact with the tissue, urea, after 3 hr of immersion, has been from 2 to 3 times that in the liver tissue immediately after its removal from the body. In these experiments water has been taken into the tissue during the first hr of immersion but later, with continued oxygenation, the increased water has usually been lost. With less opportunity for oxygen to diffuse throughout the medium (high bubbling) water intake has increased continuously during several hours. Later experiments, made under the same conditions but with liver slices in Krebs-Ringer fluid buffered with sodium bicarbonate and exposed to oxygen 95 per cent and carbon dioxide 5 per cent, had similar results but showed that more urea was formed in the presence of carbon dioxide than in oxygen alone.

More constant results have been obtained when liver slices are immersed in fluid with wide surface exposed to streaming gas and agitated by rhythmic shaking. Flasks with flat bottoms (Erlenmeyer) and capacity of 90 cc have contained 25 cc of fluid. Shaking has been at the rate of 130 per min.

The average urea content of liver slices in fourteen experiments (Table 4) has been 33.7 mg per 100 gm. of tissue with variation from 14.9 to 50.1 mg. After 3 hr of immersion, increase of urea has been approximately 3 to 6 times that of the tissue before immersion, and after 4 hr as much as 13 times that of the control, the average increase being 113.3 mg per 100 gm. of tissue and with variation from 61 to 329 mg.

Urea leaves the slices and their urea content has diminished with the progress of immersion (not shown in Table 4). In four experiments the average urea content of the liver tissue has been 37.3 mg per 100 gm. and after 60 min of immersion has decreased to 11.2 mg. It has later remained little changed, being after 120 min, 10.4 mg, after 180 min, 8.1 mg and after 240 min, 9.6 mg.

Total increase of urea (in tissue slices and immersion fluid) has been accompanied by some water intake during 60 min, but water has later been withdrawn from the the liver slices and its diminution has been continuous.

The formation of urea in the presence of oxygen and carbon dioxide (Table 4) has been compared with that which occurs with anoxia (Table 5) produced by the sub-

TABLE 4

UREA FORMATION AND WATER MOVEMENT IN LIVER SLICES WITH OXYGENATION

	Urea					Water				
Exp.	Before immersion mg	After 60 min mg	After 120 min mg	After 180 min mg	After 240 min mg	Before immersion %	After 60 min %	After 120 min %	After 180 min %	After 240 min %
1	38.2	79.0	....	137.3	....	100.0	99.8	....	95.0	....
2	14.9	67.0	80.7	102.1	....	100.0	109.3	108.3	103.4	....
3	19.2	82.2	180.9	129.0	258.3	100.0	104.5	99.2	105.0	94.9
4	38.7	...	87.6	97.3	....	100.0	....	97.6	99.3	....
5	36.4	...	82.1	99.3	111.4	100.0	....	96.3	85.0	85.3
6	26.9	61.3	....	76.8	85.9	100.0	98.1	....	88.5	86.0
7	34.9	89.9	....	121.3	132.5	100.0	109.0	....	101.2	104.7
8	31.7	68.5	78.9	90.2	100.0	100.0	97.5	94.7	97.1	83.1
9	34.8	78.5	94.2	110.6	....	100.0	112.0	110.0	110.5	....
10	26.9	62.9	75.0	89.5	....	100.0	106.0	101.3	99.9	....
11	32.8	86.2	119.0	134.2	136.8	100.0	107.1	99.7	98.3	102.8
12	45.3	58.5	116.5	143.5	....	100.0	102.1	102.3	96.8	....
13	50.1	...	137.8	....	186.9	100.0	....	107.1	....	94.9
14	40.5	...	86.0	....	164.5	100.0	....	80.6	....	82.6
Av	33.7	73.4	103.5	110.9	147.0	100.0	104.5	99.7	98.8	93.0

Liver slices have been immersed in Krebs–Ringer solution with bicarbonate buffer in flasks (with 25 cc) gassed with oxygen 95 per cent and carbon dioxide 5 per cent. Numbers are given to the experiments without relation to the order in which they were made.

TABLE 5

UREA FORMATION AND WATER MOVEMENT IN LIVER SLICES WITH ANOXIA

	Urea					Water				
Exp.	Before immersion mg	After 60 min mg	After 120 min mg	After 180 min mg	After 240 min mg	Before immersion %	After 60 min %	After 120 min %	After 180 min %	After 240 min %
6	26.9	37.4	...	40.5	35.5	100	121.3	....	105.8	113.8
15	39.4	46.9	62.2	47.7	49.4	100	122.3	129.9	129.1	129.0
13	50.1	...	67.1	...	65.4	100	....	132.7	....	130.6
14	40.5	...	53.3	...	54.8	100	....	107.0	....	112.8
Av	39.22	42.15	60.86	44.1	51.27	...	121.8	123.2	117.5	121.6

Liver slices have been immersed in Krebs-Ringer solution gassed with nitrogen 95 per cent and carbon dioxide 5 per cent, but otherwise under conditions the same as those of experiments recorded in Table 4. Experiments with the same numbers as those of Table 4 were in each instance made with liver slices from the same animals.

stitution of nitrogen for oxygen but otherwise under the same conditions. Some urea formation occurs when the medium is gassed with nitrogen and carbon dioxide but it is considerably less than that with oxygen and carbon dioxide, the quantity per 100 gm. of tissue increasing with oxygenation from 34.8 mg per 100 gm. of tissue to 134 mg after 4 hr, whereas similar increase with anoxia in the presence of nitrogen has been from 39.2 to 51.3 mg. With anoxia in the presence of nitrogen and carbon dioxide, water increase has been approximately 20 per cent but with oxygenation, after scant increase during one hr water has been lost by the slices.

A solution of the amino acid arginine (Table 3) is in water equilibrium with liver slices when its molar concentration is somewhat less (0.25 molar) than that of a sodium chloride solution (0.34 molar) isotonic with liver. Of two substances related to arginine and concerned in the formation of urea, one ornithine, is isotonic with liver slices in solutions with molar concentrations the same as that of arginine and the other, citrulline, in somewhat more concentrated solution. Substances with the osmotic properties of arginine, ornithine, and citrulline accumulating in the cell would draw water into it but in the presence of adequate oxygenation these substances form urea which, being rapidly diffusable, leaves the cell as soon as its concentration exceeds that of the surrounding medium. With anoxia, on the contrary, nitrogenous products of metabolism may supplement the pressure maintained by ions of potassium, sodium, and other electrolytes.

With the formation of urea from arginine and related substances in view, amino acid nitrogen has been measured in order to determine its changes with oxygenation and with anoxia. Amino acid nitrogen of immersion fluids with the homogenized tissue slices has been measured by the method of Folin,[8] comparing color changes with sodium beta-naphthoquinonesulphonate with those of glycine used as a standard. The precautions recommended by Natelson[9] have been followed. Protein has been removed from homogenates of tissue slices and from immersion fluids by precipitation with tungstic acid. Amino acid nitrogen has been measured in mg per 100 gm. of the immersed tissue slices. In some instances the slices have been homogenized and amino acid nitrogen of slices and of immersion fluid measured separately.

TABLE 6

AMINO ACID NITROGEN IN LIVER SLICES WITH OXYGEN AND WITH ANOXIA

	Amino acid nitrogen per 100 gm. of liver tissue				
	Before immersion mg	After 60 min mg	After 120 min mg	After 180 min mg	After 240 min mg
With oxygen	43.8	51.9	58.6	78.2	96.7
With anoxia	47.2	71.8	67.1	82.3	86.1

Liver slices have been immersed in Krebs-Ringer solution and gassed with oxygen and carbon dioxide as indicated in Table 4 or with nitrogen and carbon dioxide as in Table 5. Averages for oxygenation were obtained from six experiments and those for anoxia from four experiments.

Amino acid nitrogen in liver slices (Table 6) has been, in average, 43.4 per 100 gm. with variation from 26.9 to 64.7 mg. Corresponding figures for urea (Table 4) have been 33.7 mg per 100 gm. with variation from 14.9 to 50.1 mg. When the medium is gassed with oxygen in the presence of carbon dioxide, amino acid nitrogen in tissue slices and fluid medium increases continuously at a time when urea is increasing, but at a much slower rate, so that its quantity is slightly more than doubled. During the first 60 min of immersion the amino acid nitrogen of the

tissue slices diminishes rapidly and later maintains an even level. With anoxia in the presence of nitrogen and carbon dioxide, amino acid nitrogen has increased during four hr but the increase has been somewhat less than that with oxygenation.

Urea formation by liver tissue has been compared with that by kidney cortex under the same conditions. In Table 7 are the average results of three experiments

TABLE 7

UREA FORMATION AND WATER MOVEMENT IN SLICES OF KIDNEY CORTEX WITH OXYGEN AND WITH ANOXIA

	Urea—mg per 100 gm kidney cortex			Water—% of weight		
	Before immersion	After 120 min	After 240 min	Before immersion	After 120 min	After 240 min
With oxygen	82.4	94.6	97.3	100	130.3	133.2
With nitrogen	82.4	96.4	105.3	100	138.3	139.7

Urea formation and water movement of kidney slices under conditions similar to those recorded for liver in Table 6. Average figures are from three experiments in each of which corresponding slices have been from the same animal.

in each of which slices of kidney cortex from the same animal have been exposed to oxygen, 95 per cent with carbon dioxide 5 per cent, and to nitrogen with carbon dioxide. The average quantity of urea in slices of kidney cortex has been much greater than that in liver, i.e., an average of 33.7 mg per 100 gm. in liver (Table 4) and 82.4 mg in kidney. The table shows that little, if any urea formation has occurred. Intake of water by kidney cortex has been greater than that of liver under similar conditions and, in contrast with liver, that with oxygenation and with anoxia has been almost the same.

The experiments that have been described give evidence that liver slices immersed in a suitable medium (Krebs-Ringer solution) at 38°C, with inadequate oxygenation or with anoxia, take up water in considerable quantity increasing during 3 or 4 hr. On the contrary, with sufficient oxygenation aided by the presence of carbon dioxide, water taken in within the first hr is later in part or wholly lost. The attempt has been made in these and in earlier experiments to correlate the movement of water with changes in intracellular osmotic pressure.

Special consideration has been given to the osmotic pressure maintained by liver cells immediately after their removal from the body, for, as the result of this intracellular pressure, liver cells are isotonic with a solution of sodium chloride with molar concentration twice that of physiological salt solution or of blood plasma. The increased level of isotonicity shown by autolysis of liver when tested in graded solutions of sodium chloride has suggested that disintegration of protein may produce substances with smaller molecular weight capable of supplementing osmotic pressure referable to electrolytes, chiefly those with potassium or sodium base. Liver cells during a short supravital period act as efficient osmometers[1] and are found to be isotonic with solutions of some nitrogeneous metabolites in molar concentration which approximates that of sodium chloride isotonic with these cells.

Tests by means of graded solutions have shown that liver cells are isotonic with solutions of choline, arginine, and guanidine in concentrations approximating those of sodium chloride isotonic with liver. It is perhaps significant that ornithine and citrulline, associated with arginine in the production of urea (Table 3), are isotonic with liver in concentrations approximating that of arginine. Urea, however, is isotonic with liver only in highly concentrated solutions (approximately 1.5 molar) and, being highly diffusible, readily leaves the cell.

The present experiments show that surviving liver cells with adequate oxygenation produce urea from substances present in liver cells whereas with anoxia little urea is formed. In the former instance, loss of water is associated with urea formation. When, on the contrary, urea formation is inhibited by anoxia, there is abundant intake of water. It is noteworthy that the liver slices of these experiments contain abundant amino acid nitrogen and this increases with incubation when oxygenated, and with anoxia.

Kidney cortex unlike liver tissue under corresponding conditions forms very little urea with oxygenation or with anoxia, and water intake considerable with both, is not inhibited by oxygenation.

Evidence has been presented to show that intracellular osmotic pressure is maintained not only by electrolytes with sodium or potassium base but by nitrogenous products of metabolism as well. On the one hand, with inadequate oxygenation nitrogenous substances which increase osmotic pressure accumulate in the cell and, on the other, with adequate oxygenation they contribute to the formation of urea which is readily diffusible and leaves the cell.

Accumulation of substances which maintain intracellular osmotic pressure has been associated with the entrance of water into the liver cell. With formation of urea and presumably with diminished osmotic pressure, water has left it. This zone of osmotic activity, represented *in vitro* by adequate oxygenation at one extreme and by anoxia at the other, is, during life, favorable to the adjustment of functional needs.

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THE INCORPORATION OF LEUCINE-C^{14} INTO PROTEIN BY A CELL-FREE PREPARATION FROM MAIZE KERNELS

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In investigations of the biochemical mechanisms involved in gene-induced changes in the enzymes that regulate starch biosynthesis[1] in maize endosperm, it would be desirable to have available a cell-free system from maize endosperm that is capable of synthesizing these proteins. The present communication is a report of attempts to develop such a system. Although net protein synthesis has not yet been achieved, we have found a preparation that actively incorporates radioactive leucine into protein. This preparation has many characteristics in common with the well-known microsomal system from rat liver.[2] A preliminary report of this work has appeared.[3]

Materials and Methods.—Immature ears of waxy maize in the late milk stage were obtained from the crossing field of Dr. Drew Schwartz of this Laboratory. After harvest, all handling was conducted in a cold room at 3°C. The ears were husked and the whole kernels removed with a razor blade or sharp knife. Kernels were ground in a manner similar to that described by Raacke,[4] about 100 g in a cold mortar with 100 g of grinding sand and about 90 ml of 0.45 *M* sucrose in 0.1 *M* potassium phosphate buffer, pH 7.5. The kernels were ground vigorously for about 5 minutes until only a few small fragments of unground pericarp were still obvious. After filtration through three layers of coarse cheesecloth, the homogenate was centrifuged for 10 minutes at 1,000 × *g* in a Lourdes refrigerated centrifuge. The pellet, containing starch and cell debris, was discarded and the supernatant was centrifuged for 15 minutes at 10,000 × *g*. The supernatant solution was carefully removed with a large syringe and the pellet discarded. This supernatant was centrifuged at 100,000 × *g* for 60 minutes. The supernatant was carefully removed and saved for later fractionation. The pellet was carefully swirled in 1 ml of distilled water until free from the tube and then gently homogenized and made to a volume of 12 ml with distilled water. Any insoluble material was removed by centrifugation at 10,000 × *g* for 15 minutes; the supernatant was used for most of the experiments. When "washed" particles were prepared, the suspension was centrifuged at 100,000 × *g* for 60 minutes; then the pellet was resuspended in distilled water and, after homogenization, was centrifuged at 100,000 × *g* for 60 minutes. Resuspension and centrifugation were repeated. Finally the pellet was resuspended, homogenized, and suspended in 12 ml of distilled water.

The pH 5.2 fraction was prepared from the original 100,000 × *g* supernatant by adjustment to pH 5.2 with 2 *N* acetic acid. The precipitate was collected by centrifugation at 10,000 × *g* for 15 minutes. The pellet and tube were gently washed with distilled water, and then the pellet was dissolved in 0.05 *M* potassium phosphate buffer at pH 7.4.

The individual incubation mixtures are given for each experiment in the accompanying tables and figures. Incubations were carried out at 37°C in 10-ml flasks, with shaking. Reactions were stopped by pipetting 1 ml of 10 per cent

trichloroacetic acid (TCA) into the flask. Immediately before this, 0.1 ml of 0.1 *M* nonradioactive leucine was added to the contents of the flask.

The TCA precipitates were washed with cold 5 per cent TCA, then twice with hot 5 per cent TCA (in boiling water bath for 10 minutes), and twice with an ethanol:ether mixture (1:1, v/v). The precipitates were dried in an oven at 80°C and then solubilized in 0.2 to 1.0 ml of 80 per cent formic acid. Aliquots were taken for counting radioactivity and for measuring protein by the method of Lowry *et al.*,[5] crystalline bovine plasma albumin being used as a standard. For counting, 0.1 ml of the solution was pipetted into a bottle containing 10 ml of the following mixture: 700 ml of toluene, 300 ml of absolute ethanol, 4 g of 2,5-diphenyloxazole, and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, and the sample was counted in a Packard liquid scintillation spectrometer.

Results.—The data presented in Table 1 illustrate the energy requirement of the maize-incorporating system. They also show the stimulation of incorporation by GTP. Thus, when both the GTP and the ATP-generating system are omitted, very little leucine is incorporated into protein. Table 2 shows the dependence of the

TABLE 1

ENERGY AND GTP REQUIREMENTS FOR AMINO ACID INCORPORATION IN MAIZE PARTICLES

Incubation Mixture	Incorporation, cpm/mg of protein Experiment A	Experiment B
Complete*	200	143
Minus ATP	143	117
Minus GTP	166	92
Minus ATP generator	54	75
Minus ATP and GTP	...	18

* Consists of 50 μmoles of phosphate buffer (pH 7.4), 10 μmoles of $MgCl_2$, 1.0 μmole of ATP, 0.3 μmole of GTP, 6 μmoles of phosphoenolpyruvate, 20 μg of pyruvate kinase, 0.05 μmole of L-leucine-C^{14} (2.32×10^5 cpm). Expt. A: 2.5 mg of protein; expt. B: 2.7 mg of protein. Total volume, 1.0 ml. Incubation: 37°C, 60 minutes.

TABLE 2

EFFECT OF RNASE, CHLORAMPHENICOL, AND EDTA ON INCORPORATION IN MAIZE PARTICLES

Expt.	Incubation Mixture	Incorporation, cpm/mg of protein	Percentage Inhibition
C	Complete*	93.6	
	+ Chloramphenicol, 200 μg	69.1	27
	+ RNase, 400 μg	0.0	100
D	Complete	683.5	
	+ Chloramphenicol, 400 μg	261.7	61.6
	+ RNase, 400 μg	16.6	97.6
	− $MgCl_2$	20.7	96.5
E	Complete	356.0	
	+ EDTA, 20 μmoles	18.3	94.7
	+ EDTA, 40 μmoles	32.0	91.1

* As in Table 1, except: Expt. C—0.05 μmole of L-leucine-C^{14} (2.32×10^5 cpm), 1.2 mg of protein. Expt. D—0.05 μmole of DL-leucine-C^{14} (4.64×10^5 cpm), 0.9 mg of protein. Expt. E—0.05 μmole of DL-leucine-C^{14} (4.64×10^5 cpm), 1.0 mg of protein. Incubation: 37°C, 30 minutes.

system on Mg^{++}. These requirements are similar to those reported for the rat liver microsomal incorporating system.[2]

The time course of the incorporation process is shown in Figure 1. Incorporation is a rapid process and, like the comparable system from rat liver, the reaction is over in 30 minutes. There is no apparent explanation for the reaction's stopping at this time. In one experiment the particles were separated before the protein

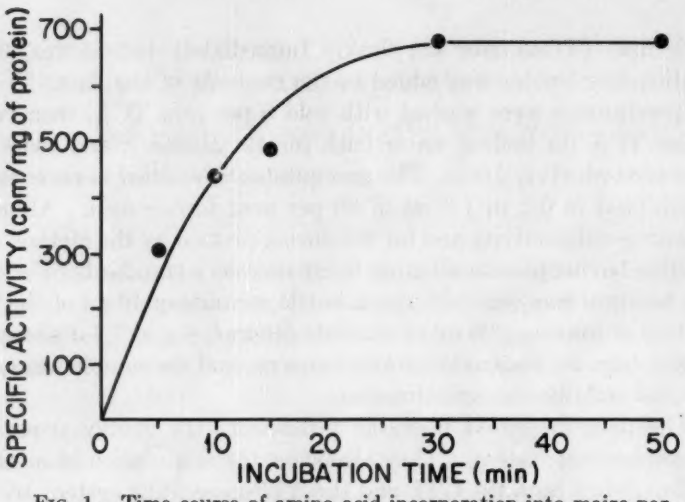

FIG. 1.—Time course of amino acid incorporation into maize particles. Potassium phosphate buffer; incubation conditions as for Table 1, except that 0.05 μmole of DL-leucine-C^{14} (4.64×10^5 cpm) was added.

was worked up for determination of radioactivity; 85 to 90 per cent of the incorporated leucine was still attached to the particles. This suggests that the particles may have a limited number of sites available for incorporation and perhaps the cessation of incorporation may be a consequence of filling the limited sites and the absence of an enzyme or factor to remove the newly formed protein.

As with all amino acid incorporation systems, we are always faced with attempting to distinguish between a process reflecting protein synthesis and one that may involve exchange reactions of one sort or another. In the absence of net protein synthesis, there is only indirect evidence for linking the incorporation system with protein synthesis. Such indirect evidence is presented in Table 2. It can be seen that the incorporation of leucine into maize protein is completely abolished by treating the system with ribonuclease. Chloramphenicol at 200 μg/ml caused a 27 per cent inhibition of incorporation and 400 μg/ml caused a 62 per cent inhibition. Because chloramphenicol is known to inhibit protein synthesis in bacteria, inhibition of incorporation of amino acids by chloramphenicol is often used as an argument that the incorporation process is a reflection of protein synthesis. Such an interpretation becomes questionable when the chloramphenicol concentration required to inhibit the incorporation process is 10 to 20 times (as in the present case) as great as that required to inhibit protein synthesis in bacteria. The recent observation that chloramphenicol at 2,200 μg/ml (100 times the concentration required for bacteria) inhibits the incorporation of amino acids into calf thymus nuclei[6] and leads to the accumulation of amino acids bound to RNA—the same point of inhibition of bacterial systems[7]—suggests that inhibition by chloramphenicol, regardless of concentration, may indeed reflect inhibition of protein synthesis.

The maize particle system seems to be more stable to storage than mammalian particles. The data of Table 3 show that particles can be stored at 3°C for 18 hours without much loss in activity, making it possible to work for several days with the same preparation. If particle preparations are frozen in liquid nitrogen,

they immediately lose about 50 per cent of their activity, but there is no further loss in activity on storage at liquid nitrogen temperatures for as long as 2 months. We have also observed that whole ears frozen at −20°C for as long as 1 week still

TABLE 3

EFFECT OF STORAGE ON PARTICLES AT 3°C ON INCORPORATING ABILITY

Experiment	Time of Storage	Incorporation, cpm/mg of protein
1	None	56.4
	18 hours	50.5
2	None	683.5
	18 hours	307.0
	1 week	48.5

Conditions as in Table 1, except: Expt. 1—L-leucine-C^{14}, 2.32×10^5 cpm. Expt. 2—DL-leucine-C^{14}, 4.64×10^5 cpm.

yield active preparations. On the other hand, particle preparations made from kernels stored at −20°C for 2 to 3 weeks are completely inactive. The age of the ear at the time of harvesting seems to be important in obtaining active preparations. Although the relation between age and active particles has not been completely worked out, our present information suggests that more-active preparations are obtained from ears harvested in the later stages of growth.

The experiments reported thus far were carried out with unwashed particles that did not require supplementation with any cytoplasmic component, although, as seen in Table 1, they did require a source of energy and GTP. It was of interest to determine whether the maize endosperm particles were like the pea seedling[3] particles that did not require supplementation with cytoplasmic constituents or whether they were like the liver microsomes that require the pH 5.2 fraction of the supernatant for full activity. Accordingly, the particles were washed as indicated in the method section and tested for requirements. The results of one such experiment are given in Table 4. It can be seen that washing the particles

TABLE 4

INCORPORATION BY WASHED MAIZE PARTICLES SUPPLEMENTED WITH pH 5.2 PREPARATIONS

Particles	pH 5.2 Preparation	Incorporation, cpm/mg of protein	Percentage of Control
Unwashed	None	484.8	100
Washed	None	24.5	5.3
Washed	Maize, 1.2 mg	379.2	78.2
Washed	Maize, 1.2 mg + amino acid	172.5	35.5
Washed	Maize, 4.8 mg	602.0	124.6
Washed	Rat liver, 0.4 mg	390.2	80.2
Washed	Rat liver, 1.6 mg	799.2	165.0
None	Maize, 4.8 mg	17.6	3.6

Complete system as in Table 1, except: DL-leucine-C^{14}, 4.6×10^5 cpm.

results in a loss of 95 per cent of their original activity. This activity can be largely restored by adding back a pH 5.2 fraction from either maize or rat liver. The best incorporation with 1.6 mg rat pH 5.2 represents about an incorporation of about 1.8 per cent of the added isotope. In this respect the efficiency of the maize system is comparable to the rat liver system.[2] On a protein basis, rat liver (at pH 5) seems to be superior to the comparable fraction from maize supernatant. The reason for this is not known, but it may be caused by different proportions of soluble RNA in the two preparations. Table 4 shows that a com-

plete mixture of amino acids resulted in a large suppression of the incorporation. The reason for this effect is also unknown. It may be caused by an imbalance of amino acids since, in a cell-free preparation from *E. coli*, a balanced mixture of amino acids that reflect the composition of *E. coli* protein is superior to an equimolar mixture of amino acids.

Summary.—A system from developing maize endosperm consisting of washed particles plus a pH 5.2 fraction from the supernatant that actively incorporates leucine-C^{14} into protein is described. The system requires a source of energy and GTP. The incorporation is inhibited by treatment with ribonuclease or chloramphenicol. When operating optimally, the incorporation efficiency is 1 to 2 per cent of the added isotope making this system comparable to the mammalian systems.

* Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

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*REPRESSION OF AN ACETYLORNITHINE PERMEATION SYSTEM**

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In 1952, it was reported[1] that an *Escherichia coli* mutant (blocked early in the ornithine-arginine pathway[2, 3]), after preliminary cultivation on arginine, will grow without lag on ornithine or arginine, but with a pronounced lag on an ornithine precursor, subsequently identified[2, 4] as N^{α}-acetyl-L-ornithine. A study of this lag led to the observation[5] in 1953 that in wild-type *E. coli*, arginine antagonizes, i.e., represses,[6, 7] the formation of acetylornithinase (which converts acetylornithine to ornithine[2, 4, 8]). Similar instances in other amino acid pathways were described in the same year.[9]

Further investigations with the *E. coli* mutant and a derivative thereof have shown that, on suitable mixtures of arginine and acetylornithine, these organisms give diphasic growth: in the first phase, arginine is utilized preferentially at wild-type growth rate, and in the second phase (after exhaustion of the added arginine), acetylornithine is utilized at a slower growth rate.[5, 6] Analysis of this diphasic growth behavior has now provided evidence for the existence of a repressible acetylornithine permeation system.

Materials and Methods.—Organisms: The following strains of *Escherichia coli* were used: mutant strain 39A-23,[1] which gives a growth response to acetylornithine, ornithine, or arginine; 39A-23R2, a reisolate of 39A-23; W (ATCC 9637),

the wild-type strain from which 39A-23 was derived; wild-type strain B, which is nonrepressible with respect to acetylornithinase[10] and to certain enzymes[11] occurring later in the arginine pathway; and 1S-14, which is a repressible derivative of strain B and was generously made available by Dr. L. Gorini.

Chemicals: N^{α}-Acetyl-L-ornithine was synthesized as reported earlier.[3, 4] DL-Glutamic-1-C^{14} acid and sodium acetate-2-C^{14} were obtained from the Nuclear Chicago Corporation and the New England Nuclear Corporation, respectively.

Cultivation: In all experiments, the organisms were grown anaerobically (nitrogen atmosphere) at 37°C on a glucose-salts medium, as previously described,[6] and supplemented as indicated below.

Growth-rate methods: Growth in matched Klett tubes containing suitably supplemented media was followed turbidimetrically as a function of time (cf. Vogel[6]). A Klett-Summerson instrument with red (No. 66) filter was employed; Klett-Summerson units are proportional to optical density.

Enzyme methods: For the determination of acetylornithinase levels, organisms were cultivated in 10 ml of supplemented medium in 125 ml Erlenmeyer flasks fitted with side arms of the dimensions of Klett tubes. Growth was allowed to proceed from an initial density corresponding to a Klett reading of 5 to a final density corresponding to Klett 40. The cultures were then chilled to 0°C and the bacteria were harvested by centrifugation, suspended in 1.0 ml of ice-cold 0.1 *M* phosphate buffer (pH 7) containing 1.0 m*M* glutathione, and disrupted by sonic vibration (in water-immersed plastic tubes). The resulting extracts were assayed for acetylornithinase activity, essentially as previously reported.[8] Results are expressed in terms of the enzyme unit defined earlier.[8] Protein was determined by the method of Lowry *et al.*[12]

Tracer methods: After preliminary growth on glucose-salts medium either with or without a supplement of L-arginine hydrochloride (50 μg./ml), the organisms were harvested, washed with distilled water, and suspended in fresh glucose-free salts medium to give a Klett reading of 50. Individual suspensions (40 ml) were placed in 250 ml Erlenmeyer flasks fitted with side arms of the dimensions of Klett tubes. Unlabeled acetylornithine (4 mg), unlabeled glucose (200 mg) and the desired tracer were added to each flask. The tracers used were either DL-glutamic-1-C^{14} acid (2 mg, 75 microcuries, as monosodium salt) or sodium acetate-2-C^{14} (1 mg, 25 microcuries). Incubation was carried out until a Klett reading of 70 was attained. The organisms were chilled, harvested, and subjected to the extraction, hydrolysis, paper chromatography, and counting procedures previously described.[13] The relative specific radioactivity of the arginine, proline, and glutamic acid from the bacterial protein was then computed, essentially as before,[13] on the basis of their relative molar abundance.

Results and Discussion.—Figure 1 illustrates the diphasic growth behavior of strain 39A-23 (cf. Vogel[5]). Arginine is used preferentially in the first phase: at the beginning of the second-phase growth, no arginine is demonstrable in the culture medium; addition of arginine at this time extends the first phase. It will be seen from the figure that the longer the organisms grow on arginine in the first phase, the slower is their growth rate on acetylornithine in the second phase.[14] It is inferred that *during growth on arginine, the organisms' ability to utilize acetylornithine is progressively antagonized.* Since L-ornithine (like arginine) added to sec-

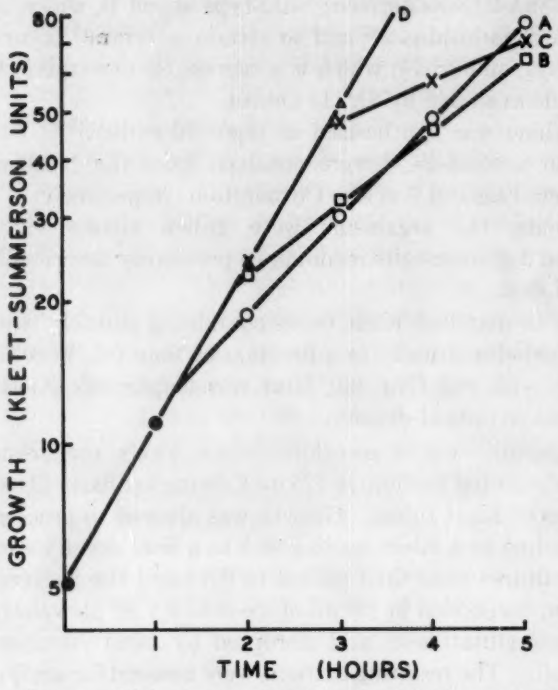

FIG. 1.—Diphasic growth of strain 39A-23 on mixed supplements of N^{α}-acetyl-L-ornithine (50 μg./ml) and L-arginine hydrochloride (A, 2 μg./ml; B, 4 μg./ml; C, 8 μg./ml; D, 50 μg./ml, excess of arginine). Solid circles represent points common to all the growth curves.

ond-phase cultures promptly accelerates the growth rate, *the antagonism to acetylornithine utilization must be directed either at the uptake of acetylornithine or at its conversion to ornithine.* Support for the former of these two possibilities has come from the following experiments.

Table 1 shows the results of growth-rate and enzyme-level studies with acetylornithine concentrations higher than those used in the preceding experiment. As the concentration of acetylornithine increases, the doubling time decreases (i.e., the growth rate increases) until, at or above concentrations of 1.0 mg per ml, the growth rate is the same as that on arginine (which is approximately the same as the growth rate of the corresponding wild-type strain when tested under similar conditions, either with or without added arginine). Table 1 also reveals that organisms growing on acetylornithine at suboptimal rates

TABLE 1

GROWTH RATE OF STRAIN 39A-23R2 AND ACETYLORNITHINASE LEVEL IN SONIC EXTRACTS OF THIS ORGANISM, AS A FUNCTION OF ACETYLORNITHINE CONCENTRATION IN THE GROWTH MEDIUM*

Supplement	Concentration (mg/ml)	Doubling Time (minutes)	Enzyme Level (units/mg protein)
AcO	0.06	103	119
AcO	0.12	83	107
AcO	0.25	73	105
AcO	0.50	67	120
AcO	1.00	63	57
AcO	2.00	63	41
AcO	4.00	63	11
Arg	0.03	63	21

* Results with L-arginine hydrochloride (Arg) instead of N^{α}-acetyl-L-ornithine (AcO) in the growth medium are included for comparison.

exhibit relatively elevated acetylornithinase levels, higher even than those of the corresponding wild type when the latter is grown on glucose-salts medium without added arginine. It is assumed that the high enzyme levels shown by strain 39A-23R2 upon growth at suboptimal rates reflect relative freedom from repression by endogenous arginine (or an "active" derivative thereof).[15] When wild-type growth rate is attained at an acetylornithine concentration of 1.0 mg per ml, the acetylornithinase level is seen to be partly repressed; under these cir-

cumstances, the formation of arginine from the exogenous acetylornithine is no longer rate-limiting for growth and, presumably, an excess of arginine is produced which causes the partial repression. At acetylornithine concentrations of 2 and 4 mg per ml, increasing amounts of arginine are thought to become available and, indeed, the enzyme is further repressed. The facts that, over a region, the growth rate increases with acetylornithine concentration and that acetylornithinase levels considerably higher than those of the wild type are reached under conditions of suboptimal growth rate indicate that the *peculiarities of utilization of acetylornithine reflect a permeation phenomenon* and not an impairment in the conversion of acetylornithine to ornithine in strain 39A-23R2.[16]

Evidence that an arginine-antagonizable uptake mechanism for acetylornithine exists not only in the mutant strain studied, but also in wild-type *E. coli* has been obtained in isotope experiments. Since the acetylornithine molecule has several functional groups and is metabolizable, "isotopic competition"[13, 17] was used as a criterion of permeation behavior. It was previously shown that acetylornithine exhibits isotopic competition in *E. coli*:[17] when labeled glutamic acid or a suitable labeled glutamic acid precursor is supplied to *E. coli* under appropriate conditions, the arginine in the bacterial protein will be labeled (at approximately the same specific activity as will be the protein glutamic acid and proline); if unlabeled acetylornithine is supplied in addition, the labeling of the protein arginine will be decreased. It has now been found, as illustrated in Table 2, that *isotopic competition by acetylornithine is antagonized as a consequence of preliminary cultivation of the organisms in the presence of arginine.* For example, after prior growth *without* arginine, in the case of glutamate-1-C^{14} as tracer, the relative specific radioactivity of protein arginine is 69 per cent, which is equivalent to a contribution by the unlabeled acetylornithine of 31 per cent; however, after prior growth *with* arginine (for either tracer), the contribution of acetylornithine to protein arginine is largely, if not completely, eliminated.[18] In contrast, the relative specific radioactivity of protein proline is not appreciably affected by prior growth of the organisms on arginine.

TABLE 2

EFFECT OF UNLABELED ACETYLORNITHINE ON THE INCORPORATION OF C^{14}-TRACERS INTO PROTEIN AMINO ACIDS, AS A FUNCTION OF PRELIMINARY CULTIVATION OF STRAIN W IN THE ABSENCE OR PRESENCE OF ARGININE*

Supplement in Preliminary Cultivation	Tracer	Protein Glutamic Acid	Protein Arginine	Protein Proline
None	Glutamate-1-C^{14}	100	69	98
Arginine	Glutamate-1-C^{14}	100	95	94
None	Acetate-2-C^{14}	100	78	93
Arginine	Acetate-2-C^{14}	100	101	93

* The results are expressed as per cent specific radioactivity relative to the respective protein glutamic acid values.

Similar isotopic competition experiments were performed with strain B, which is nonrepressible with respect to various enzymes in the arginine pathway, and with strain 1S-14, which is a repressible derivative[19] of strain B. Preliminary results indicate that prior growth on arginine does not appreciably affect the acetylornithine uptake behavior of strain B; strain 1S-14, on the other hand, resembles strain W in its uptake behavior.

It is suggested that the inferred arginine-antagonizable permeation behavior of strain 39A-23R2 and W, like arginine-antagonized acetylornithinase formation,

represents a repression phenomenon. The substance whose formation is repressed may well be a permease[20] of protein character. The preliminary results with strain 18-14 are consistent with the notion of a repressible acetylornithine permease and, in the light of the behavior of strain B, support the view that the repression of the presumed acetylornithine permease goes hand in hand with the repression of enzymes of the arginine pathway.

The presumed repression of a "constitutive" acetylornithine permease would seem to have a counterpart in the elicitation of an inducible β-galactoside permease.[20] It is known that β-galactoside permease and the enzyme β-galactosidase can be elicited by the same inducer,[20] and strong evidence is available that the induction of β-galactosidase is brought about by an antagonism of the inducer to an endogenous represser of this enzyme.[21] Acetylornithine permease under arginine-repressed conditions would thus correspond to β-galactoside permease under noninduced (i.e., endogenously repressed) conditions.[22]

General relationships between enzyme repression and induction have been formulated in terms of a unified hypothesis suggested by the acetylornithinase case: repression would involve the binding of a newly synthesized enzyme molecule to the site of its formation through the agency of the represser (or its "active" derivative) and induction would involve the neutralization of such a binding effect.[6, 7] It appears probable that the repression of permeases proceeds via the same general mechanism as does the repression of other proteins. A lucid discussion of enzyme repression and induction as cellular control mechanisms has recently been presented by Magasanik *et al.*[23] The repression phenomenon now described may be viewed as a regulatory device that, in conjunction with related known ones, provides flexibility for the cell and tends to preserve its resources.

Summary.—Studies of an *Escherichia coli* mutant blocked early in arginine synthesis indicated that this organism's restricted growth rate on acetylornithine reflects an uptake phenomenon.

Evidence has been presented that *E. coli* has an acetylornithine permease whose formation is repressible by arginine (or an "active" derivative thereof). This evidence comes (*a*) from studies of the mutant's growth rate and acetylornithinase level, as a function of acetylornithine concentration and (*b*) from isotope experiments with the corresponding wild type and with certain other strains.

It has been suggested that permeases resemble other proteins in the mechanism of their repression.

The excellent technical assistance of Mrs. Penelope K. Hait and of Mrs. Helen Carnevale is gratefully acknowledged.

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[14] It is noteworthy that one of the second-phase curves (*A* in Fig. 1) crosses the other two second-phase curves. The second-phase growth rates, therefore, do not depend on the density of the culture *per se*, but rather on a cellular property which appears to be set at the beginning of each second phase.

[15] An incisive analysis of enzyme repression by an endogenous substance has recently been presented by Gorini, L., and W. K. Maas, *Biochim. Biophys. Acta*, **25**, 208 (1957) and in *The Chemical Basis of Development*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1958), p. 469.

[16] Interestingly enough, this strain can grow at wild-type rate even when acetylornithinase is greatly repressed (see Table 1).

[17] Vogel, H. J., P. H. Abelson, and E. T. Bolton, *Biochim. Biophys. Acta*, **11**, 584 (1953).

[18] In connection with these isotope experiments, it may be noted that, in addition to acetylornithine, enzymes occurring later (cf. ref. 15) and earlier (Albrecht, A. M., and H. J. Vogel, *Federation Proc.*, **19**, 2 (1960)) in the arginine pathway of *E. coli* are known to be repressible by arginine. On the assumption of constant supply conditions of both endogenous and exogenous acetylornithine, and *ceteris paribus*, the level of acetylornithinase and of the later enzymes would not be expected to affect isotopic competition with respect to protein arginine. The level of enzymes preceding acetylornithinase in biosynthetic sequence, however, may conceivably influence isotopic competition, in view of a possible effect on the supply of endogenous acetylornithine. If, so, such influences would be expected to be quantitatively minor under the experimental conditions used.

[19] Gorini, L. (personal communication).

[20] Rickenberg, H. V., G. N. Cohen, G. Buttin, and J. Monod, *Ann. Inst. Pasteur*, **91**, 829 (1956); Cohen, G. N., and J. Monod, *Bacteriol. Rev.*, **21**, 169 (1957).

[21] Pardee, A. B. F. Jacob, and J. Monod, *Compt. rend. Acad. Sci. Paris*, **246**, 3125 (1958) and *J. Molec. Biol.*, **1**, 165 (1959); Pardee, A. B., and L. S. Prestidge, *Biochim. Biophys. Acta*, **36**, 545 (1959).

[22] Of relevance to this discussion is the elegant analysis of a "memory effect" (associated with the β-galactoside system) by Cohn, *M., Bacteriol. Rev.*, **21**, 140 (1957) and by Cohn, M., and K. Horibata, *J. Bact.*, **78**, 601, 603, 624 (1959). This memory effect is concerned with the induction of β-galactoside permease and involves the so-called preinduction (Monod, J., in *Enzymes: Units of Biological Structure and Function*, ed. O. H. Gaebler (New York: Academic Press, Inc., 1956), p. 7) or maintenance (Novick, A., and M. Weiner, these PROCEEDINGS, **43**, 553 (1957)) phenomenon. A memory effect would also appear to be implicit in the diphasic growth behavior illustrated in Figure 1, since the growth rates set at the beginning of the second phases are sustained. It seems necessary to assume that, during second-phase growth, the acetylornithine permeation system is not derepressed at an appreciable rate (although a derepression might have been expected). The present memory effect thus apparently depends on the lack of permease formation, which may have been occasioned by the relatively high bacterial density employed in these experiments. (The ultimate growth level obtained under the conditions used corresponds to approximately 100 Klett-Summerson units.) However, upon transfer of second-phase cultures

to fresh medium, especially when small inocula are involved, increased formation of acetylornithine permease appears to take place (Vogel, H. J., unpublished data).

[13] Magasanik, B., A. K. Magasanik, and F. C. Neidhardt, in *Regulation of Cell Metabolism*, ed. G. E. W. Wolstenholme and C. M. O'Connor (Boston: Little, Brown and Company, 1959), p. 334.

THE BALANCE BETWEEN COHERENCE AND VARIATION IN EVOLUTION

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What are the mechanisms that cause living things to differentiate into recognizable species and subspecific entities rather than to evolve into a flow of continuous variation? This age-old question has puzzled evolutionists, and no adequate answer has been demonstrated. One of the reasons for our highly fragmentary understanding of evolutionary processes is that only scant information has been available about mechanisms governing variation in truly wild organisms.

A recently concluded analysis of 20-year experiments on the hereditary mechanisms that separate ecological races of wild plants of western North America shows that coherence is as much a part of evolution as is variation. Even characters of evolutionary entities below the level of the species are held together by coherence mechanisms that are balanced against others producing variation. The fact that the forces of coherence are as integral a part of evolutionary mechanisms as are the forces of variation makes the process of speciation comprehensible.

Studies on the mechanisms of inheritance in wild organisms have hitherto largely been avoided because their inheritance has been known to be relatively complex. For experimental purposes, organisms that have evolved within the protective environment created by human civilization have been selected because they frequently differ in rather spectacular characters whose modes of inheritance are simpler to study. Emphasis has also been placed on the effects of individual genes as they relate to the inheritance of one or a few traits, and on the effects of mutant genes having conspicuous effects, rather than on their wild-type alleles having many small but cumulative effects.

Natural selection tends to discard the average mutant and does not directly operate on single genes, but rather upon combinations of characters regulated by constellations of genes. Races of wild plants are products of natural selection acting through geologic periods of time. They are adjusted to living in arrays of distinct environments and differ by many traits, both morphological and physiological.

GENETIC STRUCTURE OF WILD RACES

In an attempt to fill some of the gaps in our knowledge concerning mechanisms of natural evolution, our group has undertaken to analyze the hereditary structure

of ecological races of selected species of wild plants. Some of these are on record,[9-11, 13-16, 20-22] and others are still unpublished.

These investigations have shown that nearly every character distinguishing one ecological race from another is controlled by a small system of genes. The combined effects of all the genes of such a system can be easily noticed, but each component gene induces much less effect than genes commonly studied in the laboratory as mutants. The physiological effects of genes in wild races tend to counterbalance each other so that their expression is buffered. About 35 years ago Erwin Baur[2, 3] demonstrated that in crossings between wild and cultivated snapdragons (*Antirrhinum*), segregation for many small differences occurred, but no actual genetic analysis was then attempted.

The Coherence Mechanism.—The basic mechanism by which coherence is achieved is provided through the chromosomes. The several genes that regulate the phenotypic expression of a character, such as petal shape or anthocyanin coloration of stems and leaves, are usually located on separate chromosomes, but each chromosome carries genes that contribute to the control of many other characters as well. Some of the genes that regulate two distinct characters may thus be linked, whereas other genes governing these characters recombine freely. The hereditary mechanism that regulates the phenotypic expression of the characters operates, therefore, like a loose, partly disengaging network. If natural selection favors a particular trait of an organism, its effect is transmitted to the progeny through the chromosomes that carry the genes controlling that trait, and through these it will also be partially linked with several other distinct characters regulated by genes carried by the same chromosomes.

Potentials of Variation.—Natural races carry much greater potentials of variability than has previously been assumed. Crossings between wild races greatly increase the expression of variability. Epistatic (covering) effects of certain genes are frequent; likewise, genes of separate races may complement each other so that much hitherto unexpressed variability becomes released and subject to selection. Genes having additive, subtractive, and complementary effects on a character are frequently carried by separate races of the species and cause transgressive segregations in interracial crosses. In the case of physiological differences, such segregations may far exceed the limits found in the parental races.

Number of Genes per Character.—From evidence thus far obtained, each morphological difference has been found to be regulated by a system composed of a relatively small number of genes. In *Potentilla* and the Madiinae of western North America, the parental expressions of segregating individual characters have been approached, attained, or exceeded in F_2 populations consisting of 500 to 1,000 individuals under circumstances suggesting that systems on the order of three to six pairs of genes govern such characters.[11, 15] These findings are supported by other data previously obtained in diallel intercrossings between forms of the two wild pansy species of northwestern Europe, *Viola tricolor* and *V. arvensis*.[6-8, 15] The highly variable floral colors of wild pansies can be accounted for by assuming interactions between 11 pairs of genes, three of which also concern the development of anthocyanin in stems and leaves. The occurrence of an inconspicuous honey spot on the front of the pistil is regulated by the complementary and oppositional interactions of four pairs of genes.

The genetic regulation of certain physiological characters, such as date of flowering in *Potentilla*, is more complex, however; the F_2 segregation with regard to this trait greatly exceeded the limits of the parents. Considering the dates of flowering of the cloned F_2 individuals in three contrasting environments, it was estimated that a minimum of 20 pairs of genes regulate this character in the interracial crosses. In contrast, other physiological characters, such as winter-dormancy and frost resistance, appeared to be governed by three or four pairs of genes.

Combining data from crosses between several agricultural races of barley (*Hordeum*), Wexelsen[32, 33] found that 6 pairs of genes, having somewhat unequal effects, control the length of the internodes between spikelets of the ear. His conclusions were based on detailed genetic analyses of segregating F_2's, F_3's, and F_4's resulting from selfing, and were supported by the fact that two of the multiple genes were linked with two groups of genes governing other morphological characters located on separate chromosomes.

The estimates above refer to the minimal number of gene differences in organisms where it has been possible to conduct a careful and fairly representative analysis of interracial crosses. These estimates are far below the 100 to 200 genes per character that were suggested by J. Rasmusson[28] without substantiating data, but have been repeatedly quoted.[1, 26] The high estimates were based on statistical deductions rather than directly on observed segregations, and they assumed small, linearly additive differences. A moderate number of gene differences can, however, produce great diversity in segregation when the system contains complementary and oppositional genes, or when both parents carry a different set of genes having additive or subtractive effects.

Correlation in Inheritance.—Only few studies on correlations between segregating characters are on record, although there are many on linkages between individual genes. The calculation of correlation becomes a major operation when many segregating characters within a species are studied. The extensive study of correlations in progenies of interracial *Potentilla* hybrids[15] were facilitated through the use of punched card techniques.

Supporting evidence on correlations between characters is available from interracial hybrids within *Layia* and *Madia*;[9, 14, 15] and from subspecific hybrids of the *Solidago sempervirens* complex,[4] and *Gilia capitata*.[19] It was also reported in the subspecific hybrid of the lowland *Antirrhinum majus* × alpine *A. molle*.[3]

Strong correlations were observed between 8 segregating characters in a cross between two agricultural races of barley, one race developed in California and the other in Sweden.[32, 33] The segregations of 5 of these characters were widely transgressive.

At the interspecific level data are also available on correlations between segregating characters in two parallel hybrids of *Nicotiana*.[1, 29, 30] Many potential recombinations were presumably eliminated, because in the F_1 only 6 of the 9 parental chromosomes were fully homologous, about half of the pollen was sterile, and the number of seeds per capsule was reduced to one-third of that in the parents. The initial F_2 populations were small, and the parental expressions of the characters were not attained among the F_2's. The data suggest that strong limitations in recombination follow interspecific crosses in *Nicotiana*, contrasting sharply with the widely transgressive character of segregations following intraspecific, interracial crossings in *Potentilla* and in barley.

A recent paper on the cross *Nicotiana langsdorffii* × *N. sanderae*[31] strengthens the case for initial limitations in recombination and provides additional significant evidence that the tight coherences can be occasionally broken to release variation in later generations. Three selection programs were conducted among successive progenies of this hybrid until the F_{10}, aiming, respectively, for strains having short corolla tubes as in *langsdorffii*, long tubes as in *sanderae*, and intermediate tubes as in the F_1. Uniformity comparable to that of the parents was achieved by the F_6. A tube as short as that of the *langsdorffii* parent was never attained. The long corolla of *sanderae* was approached, but actually not attained, when the long-tube selection became stabilized in the F_6. In the F_9 and F_{10} generations, however, the long-tube selection suddenly surpassed the *sanderae* length in a slight, but statistically significant, transgression. Such a shift presumably could occur following a rare crossover between two genes having opposite effects on tube length and being tightly linked on one chromosome. A greater transgression in length of corolla tube was attained by backcrossing the F_5 of the plus selection to the *sanderae* parent and selecting until the F_4 following the backcross. It is therefore obvious that even interspecific crosses may, in due time, release tightly stored variability and produce transgressive variation.

EVOLUTIONARY BACKGROUND OF POTENTILLA GLANDULOSA

Potentilla glandulosa Lindl. is a semi-woody perennial species of the rose family. It belongs to an evolutionally old circumboreal complex of closely related species that was once named a genus, *Drymocallis*, but now is considered to be a section of the genus *Potentilla* and somewhat related to the strawberry. Seven species of the *Drymocallis* complex all have 7 pairs of chromosomes. The chromosome pairing is normal in interracial hybrids of *P. glandulosa*,[12] and an interspecific hybrid was fertile.[27]

Forms of *Potentilla arguta* Pursh are considered to be the immediate ancestors of *P. glandulosa*. On the North American continent, *arguta* is probably the oldest species of the *Drymocallis* complex. It occupies by far the largest area, extending from the Atlantic Ocean to the Pacific, and northward diagonally from about the 38th latitude in the east to the 60th latitude in the west.[12]

Potentilla glandulosa is more variable than *arguta*, but occupies a more limited area in the western United States essentially south of the general region of *arguta*,[12] an area that has been the scene of great mountain-building processes during the 60 million years of the Cenozoic era. During the first period of that era, the Eocene, the California part of the present *glandulosa* region is known to have had an essentially tropical vegetation; the mountains were low hills, and the Central Valley was a huge bay of the Pacific Ocean. Like the tree species of the western United States, it is assumed that the ancestors of *glandulosa* migrated from the north, and gradually occupied the new niches that became available as the climates changed from tropical to warm temperate, and as the mountains rose.

Potentilla glandulosa is the most highly diversified species of the *Drymocallis* complex, and has evolved a series of ecological subspecies and races fitting the highly diversified niches in its topographically complex area.[12, 15] There is reason to believe that the ancestors of its outer Coast Range and high-mountain races have been in position during the last 20 to 30 million years, from the beginning of the Miocene period, and that since that time the forms inhabiting the mountains have

undergone gradual selection, adjusting them to survival in the climates of the rising mountains. The climatically youngest environment in the *glandulosa* area is the warm and dry inner foothill region. It has existed only since the California Coast Ranges started their uplift and blocked the moist and cooling influence of the Pacific Ocean—an event that presumably began about a million years ago.

The differentiation into climatic races within this species has affected its breeding system. The lower altitude races are self-compatible and have small, relatively inconspicuous petals. The races of the montane to alpine altitudes of the Sierra Nevada are highly self-incompatible and have large, showy petals. Comparable changes have also taken place in the life forms of the races. The low altitude ones are woody cushion plants that have heavy tap roots and branch from the crown (chamaephytes), whereas those of high altitudes spread by slender, subterranean rhizomes and die to the ground level in the autumn (hemicryptophytes).

SEGREGATION AND COHERENCE IN POTENTILLA HYBRIDS

Two hybrids from four contrasting races of *Potentilla glandulosa* were analyzed with regard to the gene systems that regulate their differences, and the degrees of correlation between parental characters were determined among their segregating progeny. One cross was followed through the second generation in one environment. The progenies of the other cross were studied through the selfed third generation, and second generation plants of this hybrid were cloned and transplanted to three contrasting environments where their performance over many years was carefully studied.

Coast Range × Alpine Hybrids.—A cross between a plant of a coastal race of *Potentilla glandulosa* from near sea level at Santa Barbara, California, and another of an alpine race from Upper Monarch Lake, at 3,300 meters altitude in the Sierra Nevada, yielded F_1 progeny that in some characters was intermediate between the parents, in others favored one or the other parent, and in vigor exceeded both. The coastal parent was self-fertile, the alpine self-sterile, and dominance for self-fertility in the F_1 was so strong that a single selfed plant yielded nearly 30,000 good seeds. The Coast Range race is unable to survive in the alpine climate, and the alpine in the Coast Range, but the F_1 hybrids surpassed both by surviving in Coast Range, mid-altitude, and alpine gardens.

The two races differed in many morphological and physiological traits. A sample of 1,000 F_2 hybrid progeny were grown and showed a striking amount of segregation in all characters, and transgressive segregation in many, 10 per cent of the segregants even exceeding the vigorous F_1 in size. Fourteen character differences distinguishing the parents were studied with respect to their degree of correlation, or frequency of association together, in the F_2 progeny.

Figure 1 summarizes the data in the form of a diagram. It is evident that most characters are not inherited independently of each other, but are partially linked to others in varying degrees. Among the 91 possible combinations of the 14 characters studied, 67 were associated together in some degree, and 24 showed no significant correlation. The majority of parental characters were therefore directly or indirectly correlated, although also a great deal of genetic recombination occurred among all of them. Probably all of these characters are either directly or indirectly related to the success of the races in their respecitve environments.

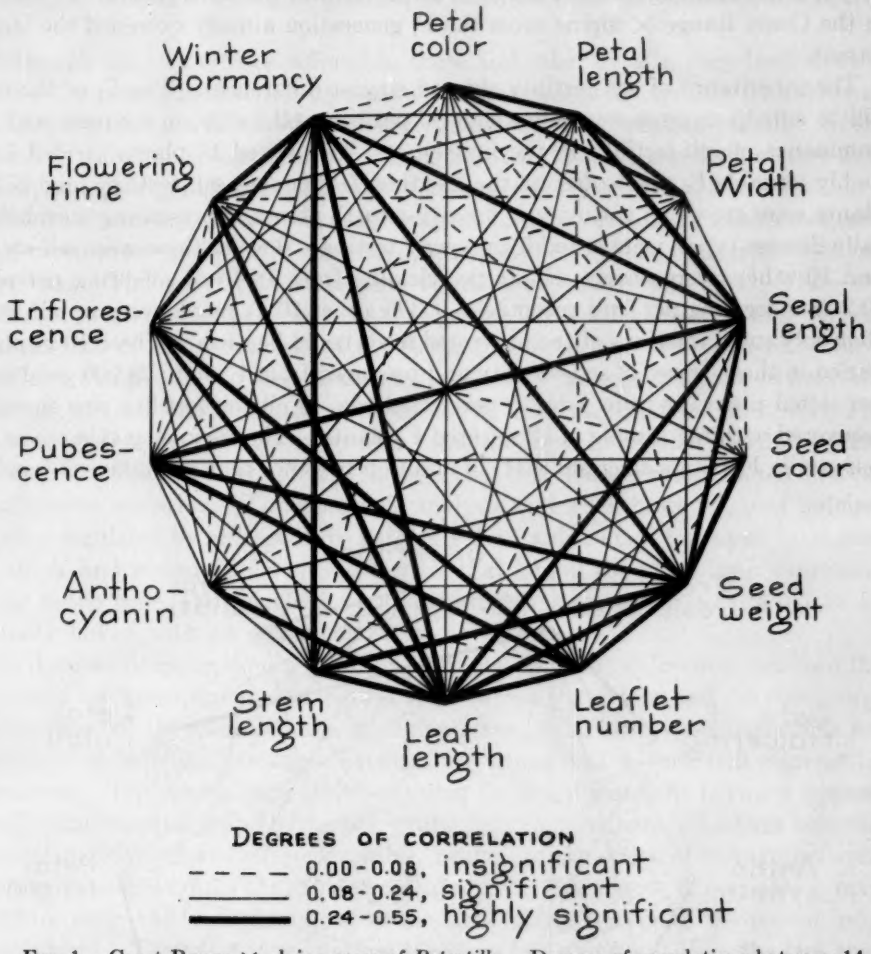

FIG. 1.—Coast Range × alpine races of *Potentilla*. Degrees of correlations between 14 segregating characters in an F_2 progeny of approximately 1,000 individuals. Correlation coefficients, *r*, above the 1 per cent level (0.08) considered significant.

Foothill × Subalpine Hybrids.—A second hybrid was a cross between a plant of the foothill race from Oak Grove in the Sierra Nevada foothills at 760 m and another of the subalpine race from a slope above our Timberline station situated in a hanging valley on the east side of the crest of the Sierra Nevada at 3,050 meters altitude. The contrasts in characters between the parents of this hybrid were fully as great as between those of the former, and there were certain significant differences between the two crosses, especially in the expression of hybrid vigor and in the inheritance of self-fertility.

The F_1's of the foothill × subalpine cross exceeded only slightly in size the intermediate position between the parents, and almost all of the F_2 plants varied between the ranges of the parents. Six of the selfed F_3 progenies, however, showed luxuriance of growth to the extent that 45 per cent of their 2,228 plants surpassed the tall foothill parent; three other F_3 progenies showed minor degrees of hybrid vigor. The genetic potentials for hybrid vigor therefore existed in this cross, but the

proper gene combinations had not been achieved until the third generation, whereas in the Coast Range × alpine cross the F_1 generation already exceeded the largest parent.

The inheritance of self-fertility showed similar differences. The F_1 of the foothill × subalpine cross was only about 40 per cent self-fertile, in contrast with the dominance of self-fertility in the first cross. Two selfed F_1 plants yielded 1,070 highly variable F_2 plants despite the less than 50 per cent self-fertility, and 575 F_2 plants were grown to maturity. Twenty-two F_2 plants, representing morphologically diverse types, were selfed for progeny testing. Two of these were self-sterile and 10 others were nearly self-sterile, yielding from 16 to 57 offspring per plant (0.2 to 1 seedling per fruit receptacle). The other 10 F_2 plants were so self-fertile that only small fractions of the harvested seeds could be sown. These 10 F_2 plants varied in their degree of self-fertility and produced from 1,200 to 28,000 good seeds per selfed plant, or from 4 to 25 per receptacle. Full self-fertility was therefore recovered within a sample of 22 selected F_2 plants. The frequency of more or less self-sterile F_2 plants suggests that, in addition to genes that promote self-fertility,

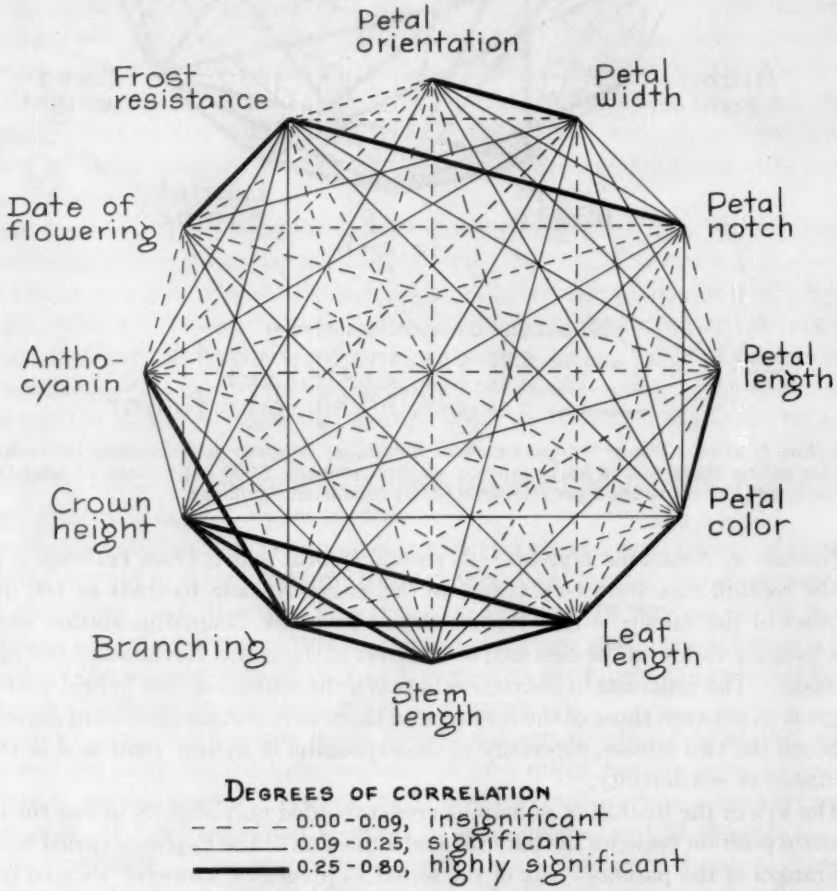

FIG. 2.—Foothill × subalpine races of *Potentilla*. Degrees of correlations between 12 segregating characters in an F_2 progeny of approximately 575 individuals. Correlation coefficients above the 5 per cent level (0.09) considered significant.

others that inhibit it were also present, in contrast with their absence in the first cross.

Although the two crosses differed in these and other details, they both demonstrated the principle that the parental characters are held together by coherence mechanisms that are balanced against great potentials for recombination. In the foothill × subalpine cross, the correlations between 12 segregating characters were tested, and the findings are depicted in the diagram, Figure 2.

The 12 characters permit 66 possible combinations of pairs of characters, and 38 of these were significantly correlated, whereas the other 28 appeared to be uncorrelated. The correlation coefficients varied between 0.10 and 0.64. These are moderate to fairly strong correlations that permit various degrees of recombination.

Almost all of the characters of the foothill × subalpine cross appear to have significance in relation to the success of the parental races in their native habitats, although at least one obviously appears not to have any such significance. The character notched petals has so far been found in only one deviant subalpine plant in the wild. For this reason, it was thought that this character represented a recent spontaneous mutation. The genotypic analysis indicated, however, that notched petals is regulated by at least three pairs of genes: a dominant but hypostatic gene for notch, and two multiple, epistatic genes that inhibit the phenotypic expression of the notch gene. Although the notch character is so rare, it was found to be partially linked with six other characters (Fig. 2).

No deep significance should be attached to the apparent differences between the degrees of coherence among the foothill × subalpine characters, and the coherences among those of the Coast Range × alpine cross. The observed correlations are resultants of individual linkages between the genes that govern two segregating characters. The correlations shift according to the interactions between oppositional, complementary, and hypostatic genes that regulate them. Linkage between genes of positive effect can, for example, neutralize the effect of linkage between negative genes governing the same two characters. The degree of correlation may therefore vary widely depending upon the particular genotype of the parent individuals used. The exact percentage is therefore less significant than the fact that the characters of races cohere and also recombine.

Selective Effects.—Moderate coherence, such as that observed in the interracial foothill × subalpine cross, has real significance for survival of the segregating progeny under field conditions. This was shown by cloning each of the 575 F_2 plants of this cross and planting a part of each at our three altitudinal transplant stations where they were exposed to the effects of contrasting environments, and the responses were studied over a period of years. The stations are: at Stanford, in central California in the oak savanna region near sea level; at Mather, in the yellow pine forest of the Sierra Nevada at 1,400 m; and at Timberline, at the upper reaches of the lodgepole pine forest at 3,000 m near the summit of the Sierra Nevada. The climates of these stations range between the mild Coast Range climate at Stanford having an all-year growing season, to the inhospitable Timberline climate where the growing season is compressed within $2^1/_2$ late summer months with relatively short days and cold nights. Detailed notes were taken on each plant over a period of 5 years at Stanford, and of 9 years at each of the mountain stations.

In order to provide a numerical scale for the degree of resemblance of each hybrid

TABLE 1

SURVIVAL PERCENTAGES OF 509 F_2 CLONES OF THE FOOTHILL × SUBALPINE CROSS OF *Potentilla* IN RELATION TO INDEX VALUES

	Station, and Length of Experiment	Classes of Index Values: 20–29 Most Subalpine-like	30–39 Intermediate	40–50 Most Foothill-like
Survival percentages	At Timberline 3,000 m, 9 years	75.6	34.3	13.5
	At Mather 1,400 m, 9 years	49.5	71.5	76.5
	At Stanford 25 m, 5 years	17.8	77.0	70.8
Number of clones		90	330	89

Index values of parents:
Subalpine..... 19
Foothill..... 54
F_1..... 34

plant to its parents, each character of the parental plants, the F_1, and all the F_2's, was rated in such a way that a low value was assigned to the expression of that character in the subalpine parent, and a high value to its expression in the foothill parent, making allowance for the F_2 segregation concerning that character. An index of value of each plant was obtained by adding the values of all 12 characters that were studied. As seen in Table 1, the index value of the subalpine parent was 19, that of the foothill parent was 54, and for the F_1 it was 34. The scale made allowance for values from 12 to 64, and those F_2 plants of which all characters could be scored varied between 22 and 50.

For the sake of simplification, the index values of the F_2 plants were arbitrarily grouped into three classes, each ranging roughly over 10 values. These were: low, 20–29; intermediate, 30–39; and high, 40–50. The index values of the plants of the low class were closest to the value of the subalpine parent, those of the high approached the foothill race, and the intermediate group clustered more or less around the value of the F_1 type. These classes are purely numerical, and do not necessarily imply superficial resemblance to a particular parent. It would have been impossible to classify the segregants by sight, because the characters were quite thoroughly shuffled.

Each cloned plant was also classified according to its period of survival at each station. The subalpine parent died repeatedly at the lowland station, but survived during all 9 years at Mather and Timberline. In contrast, the foothill parent survived for the entire experimental period at the lowland and mid-altitude stations, but consistently died promptly at high altitude. The F_1 individuals combined the tolerances of both parents and survived indefinitely at all three stations. An F_2 plant was considered to be a survivor at a particular station if it survived during the entire period of the experiment. It was listed as a nonsurvivor if it died once or twice. The percentages of only the survivors are listed in Table 1. The percentages of the nonsurvivors are 100 minus the survival percentage.

Ninety of the 509 fully classified F_2 plants are in the subalpine-like 20–29 class, and 75 per cent of the 90 survived all 9 years at the Timberline station, the other 25 per cent being nonsurvivors (Table 1). Sufficient recombinations had occurred

among the subalpine-like class so that 18 per cent of this group were able to survive the full 5 years at the lowland station.

At the other extreme, a group of 89 F_2 plants had high index values between 40 and 50, statistically approaching the value of the foothill parent. Seventy per cent or more of this group survived at the lowland and mid-altitude stations, but more than 13 per cent of the group had, through recombination, acquired the capacity to survive all 9 years in the extreme climate at Timberline.

The 330 F_2 plants having intermediate index values between 30 and 39 tended to follow the foothill parent at the two lower stations, but a fairly large number of individuals, 34 per cent, were able to survive at 3,000 meters.

It is especially significant from the evolutionary point of view that 148 F_2 plants (29.7 per cent) were able to survive at all three stations during the entire period. These plants represented a wide range of index values, although the majority belonged to the large intermediate group. None of the parents, nor any of the previously sampled plants from the wild,[12] had demonstrated such a wide range of tolerance.

These data clearly indicate that the physiological traits that enable the subalpine race to survive at high altitudes, and the foothill one at low, cohere strongly with the kind of characters that can be scored to produce index values. The coherence is nevertheless flexible enough to permit some recombined subalpine-like plants to survive at low altitudes, and some of the foothill-like segregants to live at high altitude. Likewise, the interracial crossings resulted in many recombinations that showed new growth patterns and presumably would be able to fit new niches. Many of these innovations had a widely increased range of tolerance. There is reason to believe that these findings apply to ecological races of many kinds of organisms.

IMPLICATIONS OF COHERENCE

Coherence mechanisms have not been prominent in discussions of evolution, although their existence should have been predictable. For example, at the chemical level, the significance of coherence through chemical bonds has long been recognized, as well as variations represented by the composition and arrangement of side chains. At the highly complex level of the living cell, the structures of DNA and RNA molecules exemplify coherence within molecular chains and variation as different sequences of nucleotides.

Early experimenters, such as Linnaeus,[25] Koelreuter,[24] and Gärtner,[18] knew of the strong coherences that counteract interspecific hybridization. Darwin[17] cited the experiments of the latter two authors but doubted the general significance of their findings, because he knew that orchid hybrids and willow hybrids were fertile. Darwin, in general, de-emphasized the distinction between species and varieties and emphasized that natural variation provides the materials for natural selection. Variation is, indeed, the important element in change, but distinct kinds of organisms could not have remained distinct and recognizable throughout geologic epochs without some kind of inherent restraining influence.

Alexis Jordan in 1846[23] had already shown through many experiments that even local populations of certain plant genera, for example the violets, could be

morphologically distinct and developed true from seed. Darwin does not mention Jordan's discovery, but he may have missed its significance because Jordan, following the trend of the time, named such distinct populations species. What Jordan observed was undoubtedly a combination of genetic drift[34] and genetic coherence. The coherence systems that exist at the level of the ecological race are carried by the individuals of the race, and as such are also a feature of the local populations of which races are composed.

The finely balanced genetic equilibria that make it possible for an organism to live and to adjust to its environment are so complex that many generations are required to reassemble them after they have been broken down through hybridization. The coherence mechanism protects the continuancy of the balances.

Coherence, combined with natural selection, makes it possible for two ecological races to exist in adjacent but ecologically distinct habitats without losing their identities. Races of *Potentilla* in moist, cold meadows are contiguous with others on adjacent dry, warm slopes, but they keep distinct although they are interfertile.[12, 15] So do the adjacent and interfertile annual inland, and perennial maritime sand dune races of *Viola tricolor* in west Jutland, Denmark.[5]

The arrangement whereby genes which govern a character are located on separate chromosomes provides a fair degree of resilience in the inheritance of the character, and in the adjustment of the race to minor differences in environment. From the opposite point of view, if the genic controls of characters were closely assembled on single chromosomes, recombination would involve large steps and the coherence-variation equilibrium would become impaired. It should be remembered, however, that genes located remote from each other on a chromosome can function as if they were independent of each other.

Conclusions.—The evidence available from experiments on the inheritance of ecological races indicates that the survivors throughout geologic periods have been those kinds of organisms that possess a fair degree of inherited coherence balanced against potentials of variation that can be made available through interracial crossing. It is the device of coherence that enables ecological races to function as reservoirs for potential variability.

As a result of their dynamic coherence-variation balances, natural entities remain unchanged so long as the environments remain essentially the same. Major changes in the environments cause migrations of races, interracial crossings, release of variability, and changed selective pressures. These events can lead to establishment of new races adjusted to new environments. Certain species have nevertheless been able to retain their basic coherence mechanism relatively unchanged throughout geologic periods, as has been demonstrated, for example, in the chromosomal homology of hybrids between the Old World and New World sycamores of the genus *Platanus*.[15]

Once an equilibrium between coherence and variation has become established, it adjusts to all levels of speciation, ranging from the local population to the ecological races, the species, and beyond. A species may age during its evolutionary processes, but as long as it is able to maintain enough local populations, enough ecological races, and maintain an effective balance between coherence and variation within its entities, it is still, in a biological sense, a youthful species.

The restraints of coherence become stronger as evolution proceeds toward entities of higher order. In organisms like *Drosophila*, *Oenothera*, and *Holocarpha*, inversions and other transpositions of segments of chromosomes isolate groups of genes into super-genes. These rearrangements within chromosomes increase coherence and decrease flexibility. At the level of distinct, although closely related, species the genomes have become sufficiently distinct so that interchange of their genes results in unfitness of a major percentage of the hybrid progeny, thereby increasing coherence and decreasing effective recombination, as was discussed under *Nicotiana*. A further step toward evolutionary separation is the differentiation of whole sets of chromosomes that cohere and can be added but not recombined, a step that often distinguishes subgenera or sections of a genus.

Distinct genera have lost their ability to intercross and may carry vast cores of heredity held together by unbroken coherences. Their component species and ecological races, however, represent many coherence-variation equilibria, and through them they carry vast possibilities for evolution and adjustment to new conditions.

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PARAMAGNETIC DEFECTS IN IRRADIATED $KClO_4$

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Electron magnetic resonance (EMR) studies of X-ray damaged single crystals of $KClO_4$ indicate the presence of two types of stable paramagnetic defects at room temperature. One type of defect appears to be a trapped ClO_2 molecule.

Single crystals of $KClO_4$ were grown by slow evaporation of an aqueous solution. Potassium perchlorate is orthorhombic[1] with 4 molecules per unit cell. Cell

dimensions are $a = 8.834$ A, $b = 5.650$ A, $c = 7.240$ A; the space group is V_h^{16} (Pnma). Irradiations were performed at room temperature in air with 50 kv X-rays from a tungsten target tube operating at 20 ma. After irradiations of from 10 minutes to two hours the crystals exhibited color ranging from pale yellow to brown. EMR spectra were observed with a Varian V 4500 spectrometer using a cylindrical cavity operating in the TE_{01} mode at 9536 Mc.

Figure 1 shows a typical EMR spectrum of an irradiated and aged single crystal;

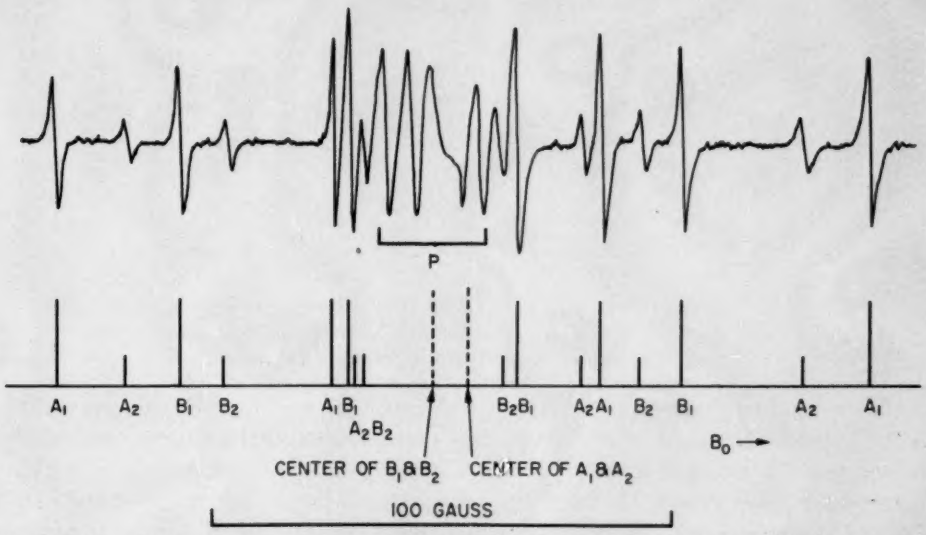

FIG. 1.—Electron magnetic resonance spectrum of irradiated $KClO_4$ crystals. The lower schematic spectrum shows the assignment of lines to the two defect sites (A and B). Subscripts 1 and 2 indicate Cl^{35} and Cl^{37} respectively.

its main features are (1) four groups labeled A_1, A_2, B_1, and B_2, each group having four lines of nearly equal spacings and (2) a group of closely spaced lines labeled P. Spacings of lines within each group as well as the relative positions of the groups depend upon the crystal orientation relative to the applied magnetic field. Immediately after irradiation only the P group was observed; thereafter the other four groups appeared and increased in intensity to the terminal value shown in Figure 1. A third set of lines appeared concurrently with the A and B groups but two days after irradiation their intensity began to decrease, becoming too weak to observe after about 10 days. Observed spectra of aged crystals can be accounted for by the following assumptions.

(*a*) The species producing group A_1, A_2, B_1, and B_2 contains only one Cl nucleus; A_1 and B_1 a Cl^{35}, A_2 and B_2 a Cl^{37}.

(*b*) There are two nonequivalent sites for the Cl associated defect. Figure 2 illustrates the orientation of the principal axes of the hyperfine tensors (x, y, z; x', y', z') of the two sites relative to the unit cell axes. These two sites give rise to the A and B groups.

(*c*) In the set of defect axes labeled x, y, z for A and x', y', z' for B the molecular spin Hamiltonian is,

$$\mathcal{H} = \beta \mathbf{S}\cdot\mathbf{g}\cdot\mathbf{B}_0 + g_n\beta_n \mathbf{I}\cdot\mathbf{B}_0 + \mathbf{I}\cdot\mathbf{A}\cdot\mathbf{S} + \mathbf{Q}\cdot\nabla\mathbf{E} \qquad (1)$$

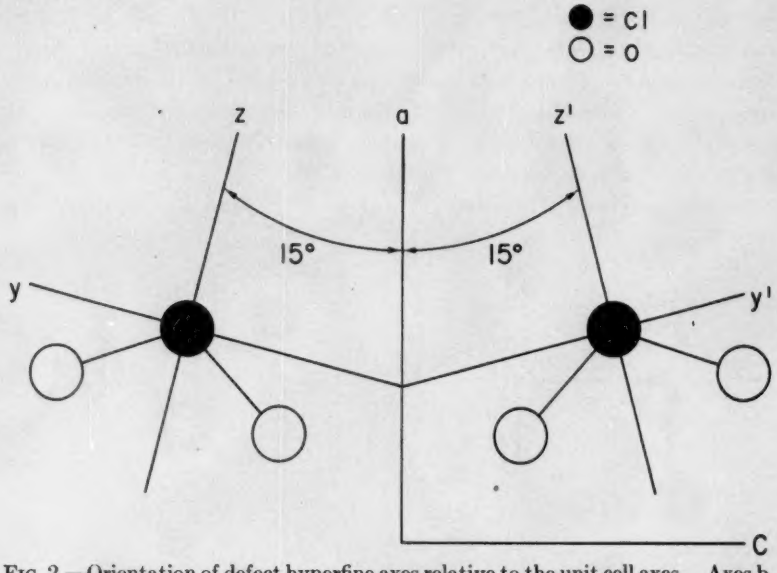

FIG. 2.—Orientation of defect hyperfine axes relative to the unit cell axes. Axes b, x, and x' are perpendicular to the plane of the figure.

$\mathbf{B}_0$ is the applied magnetic field **S** and **I** the electron and nuclear spin operators, **A** the hyperfine coupling tensor, **g** the electronic g tensor, **Q** the nuclear quadrupole tensor and $\nabla\mathbf{E}$ the field gradient tensor. β and β_n are the electronic and nuclear magetons respectively. The first and second terms in (1) are the electronic and nuclear Zeeman terms, respectively, while the third and fourth are the hyperfine and nuclear quadrupole energies. Principal values of the diagonalized A and g tensors determined for Cl^{35} were:

$$A_{xx} = 44 \pm 4 \text{ Mc} \qquad g_{xx} = 2.0183 \pm 0.0005$$
$$A_{yy} = 32 \pm 4 \text{ Mc} \qquad g_{yy} = 2.0088 \pm 0.0005$$
$$A_{zz} = 204 \pm 2 \text{ Mc} \qquad g_{zz} = 2.0036 \pm 0.0005$$

The nuclear Zeeman and quadrupole terms produced only small effects in the EMR spectra, in particular the quadrupole term was estimated to be less than 10 Mc. Diagonal values for **A** in the Cl^{37} defects were found to be smaller by a factor of 0.83 (equal to the ratio of nuclear magnetic moments). Diagonal values of **g** for Cl^{37} defects were the same as for Cl^{35} defects.

(*d*) The *P* group of lines arises from other paramagnetic species.

The identification of the Cl associated defect as ClO_2 rests on three points. First, $\frac{1}{3}|\text{Tr } \mathbf{g}|$ is 2.010 in agreement with the value found for ClO_2 in solution by Bennett and Ingram;[2] the isotropic hyperfine interaction given by $\frac{1}{3}|\text{Tr } \mathbf{A}|$ is 43 Mc, again in agreement with the value of 46.5 Mc found for ClO_2. The value of 43 Mc is obtained if A_{xx} and A_{yy} are opposite in sign to A_{zz}. Secondly, **g** and **A** being noncylindrically symmetric eliminate species such as ClO_3 and ClO. Lastly, the values given above for the diagonal components of **A** are in semiquantitative agreement with hyperfine components calculated from MO theory.[3] Further studies are in progress to determine nuclear quadrupole interactions and to identify the transient species mentioned above.

The author wishes to thank Dr. A. H. Silver of this laboratory for his enlightening discussions of this work.

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*CONTINUOUS ELECTROPHORETIC FRACTIONATION STABILIZED BY ELECTROMAGNETIC ROTATION**

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Communicated by Walter M. Elsasser, February 8, 1960

The objective of the experiments described below is to achieve continuous separation of ions in solution by making charged particles of different mobilities follow divergent paths. Similar aims have been pursued by other authors employing different means. Grassman and Hannig[1] utilized in their paper curtain electrophoresis gravitational descent of a flowing zone of the dissolved mixture of ions along a curtain of filter paper in a transverse electric field. Svensson and Brattsten[2] aimed to achieve the same result by using glass powder as a medium to suppress thermal convection. Mel[3] used a density gradient for stabilization, putting gradient zone electrophoresis[4,5] on a continuous flow basis, and Dobry and Finn[6] relied on suppression of thermal convection in a viscous vertical flow column. The present approach differs from the previous ones in that no porous media or concentration gradients are required for stabilization against thermal convection, and in the use of electromagnetic forces in combination with an electric field to obtain the desired motion of the ions in a free solution, stabilization being achieved by revolution of the electrophoretic column about a horizontal axis. The resulting stability is so great that high current densities in cooled liquid columns can be employed permitting continuous application of potential gradients in the vicinity of 50 Volts/cm and brief application of fields exceeding 100 Volts/cm. The use of very thin and sharp "streaks" of ions in strong electric fields leads to clear-cut separations within time intervals of the order of a few seconds (Fig. 10*d*). The method is applicable to suspended particles as well as to ions in solution.

The application described below is designed for separation on a micro-scale, processing mixtures at the rate of less than 0.01 cc/min. No attempt has been made as yet to construct an apparatus for large-scale separations.

Principle of the Method.—The main experimental difficulty to be overcome is thermal convection. In addition to the above mentioned methods of stabilization,[1–6] there are two stabilization principles which have not been used as yet for continuous flow separations: (*a*) Stabilization by an electromagnetic force field,[7] which has been used so far only in experiments on electromagnetophoresis, and (*b*) stabilization by rotation of the fluid[8,9] which has been used practically so far only with zone electro-

phoresis in free solution.[9] The latter method (*b*) is based on the following consideration. A heated fluid element which tends to rise in the field of gravity due to diminution of its density below that of the surrounding fluid will describe a circular motion instead of rising steadily, if the cell is rotated slowly. As a result, thermal convection does not develop even in the presence of high local density gradients of thermal origin.[8] This principle is utilized in the present method. A horizontal cylindrical fluid column of annular cross-section is maintained in rotation about its axis of symmetry by electromagnetic forces which inhibits thermal convection.

The rotation of the fluid column is produced by a combination of a longitudinal electric current with a radial magnetic field, as shown in Figure 1. The $\mathbf{B}_r$ vectors represent radial components of the magnetic field in a plane perpendicular to the axis of the bar magnet (M) at a distance R from the magnet axis. **J** represents the vector of current density in the conductive fluid surrounding the magnet and $\mathbf{f} = [\mathbf{J} \times \mathbf{B}]$ the force density, i.e., the force exerted upon each unit volume element

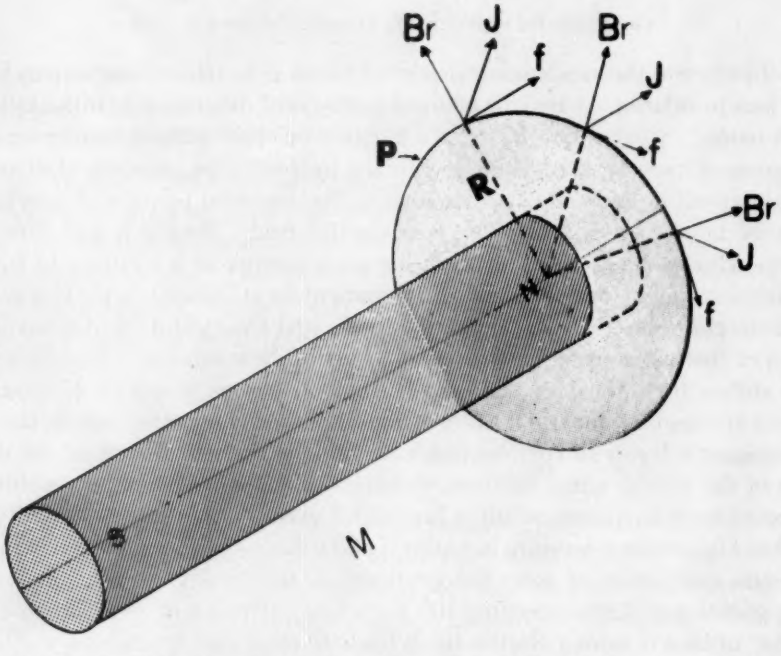


FIG. 1.—Spatial configuration of the vectors $\mathbf{B}_r$, **J**, and **f**. *N, S:* poles of the bar magnet.

at right angles to $\mathbf{B}_r$ and **J**. The circular arc P represents a path of a fluid element. Fluid particles along the periphery P will be set in rotation about the bar magnet by electromagnetic forces. In order to obtain a radial magnetic field of adequate longitudinal extent and uniformity, the opposing like poles of two bar magnets M_1 and M_2 can be bridged by an intermediate short, soft iron cylinder *m* as shown in Figures 2, 3*a*, and 7*b*. The resulting distribution of the radial component of the magnetic field intensity at the distance R from the axis is indicated in Figure 7 for a single bar magnet and for the arrangement of two bar magnets described above. The field intensity distribution was determined by means of a mercury jet magnetometer[10]

by moving the magnet past the magnetometer and recording directly the radial component of the magnetic field.

The electrophoretic column is made short enough to be penetrated only by a small section of the magnetic field of adequate radial intensity (namely, the section adjacent to the magnet poles), as shown in Figure 2. This perspective view shows an electrophoretic cell consisting of three externally discernible sections. B is the annular electrophoretic column surrounding the central channel into which the ends of

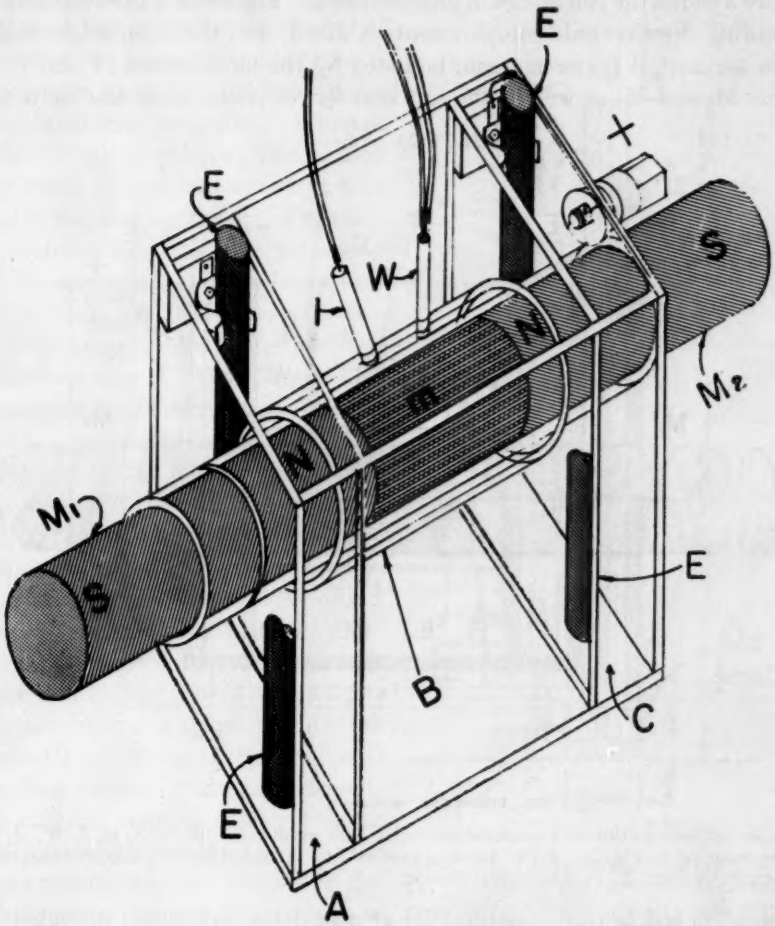


FIG. 2.—Perspective view of the fractionation cell. *N, S:* poles of the bar magnets M_1 and M_2; *m:* soft iron spacer; *E:* four graphite electrodes (the two front electrodes are shown only partly). Each electrode compartment (*A* and *C*) harbors two electrodes which are connected to a common binding post. Only the positive binding post is shown. *I:* injector from which the mixture to be fractionated enters the cell; *W:* withdrawal peg through which separated fractions are removed; *B:* the actual fractionation space surrounded by lucite walls. The tub thus formed is filled with an ice-water mixture.

the bar magnets M_1 and M_2 are inserted. *m* is the soft iron section sandwiched between the end faces of the cylindrical bar magnets M_1 and M_2. A and C are electrode compartments which are communicating with the ends of the annular electrophoretic column, and E represents graphite rod electrodes. The bottom and

the sides of the electrode compartments are bridged by lucite plates so as to create a transparent bath surrounding the section B. This bath is filled with ice-water mixture to cool the electrophoretic column. The latter is thus an annular cylindrical volume bounded on the inside by a lucite cylinder surrounding the bar magnets and on the outside by a cylinder in contact with the ice-water bath. The width of the annulus is 1.5 mm and its axial length is 6 cm. The thickness of the cylinder walls is 0.5 mm.

Figure 3 shows the cell design in greater detail. Figure 3*a* is a central longitudinal cut showing the electrode compartments A and C and the annular electrophoretic column segment B between them, bounded by the lucite tubes IT and ET. The magnets M_1 and M_2 as well as the soft iron spacer *m* are inside the lucite tube IT.

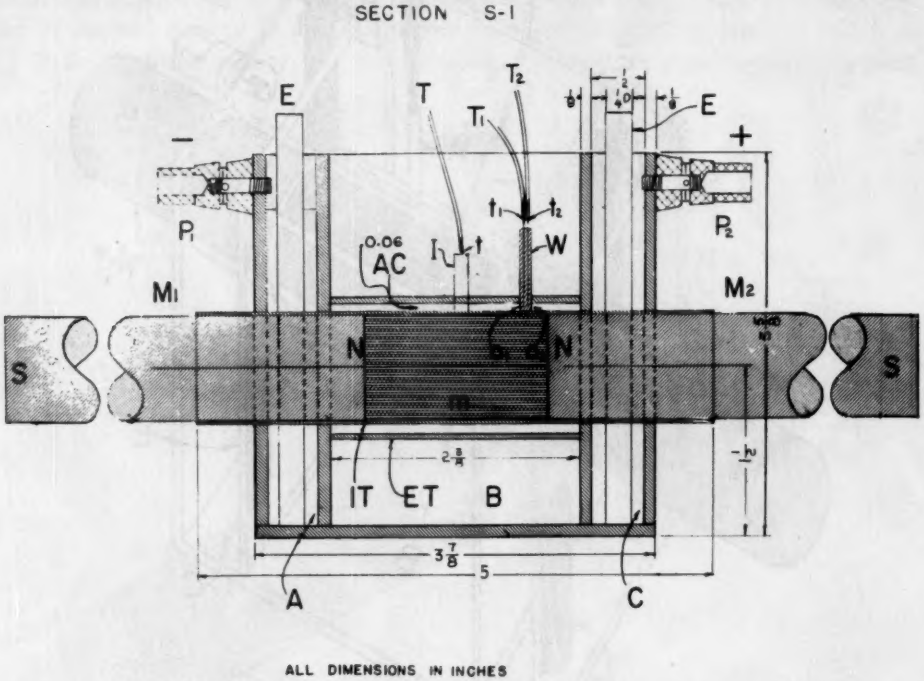


FIG. 3*a*.—Cross-section of fractionation cell. The significance of *N*, *S*, *m*, *I*, *W*, *A*, *C*, *B*, and *E* is the same as in Fig. 2. P_1P_2: binding posts; *AC:* annular electrophoretic channel; *t*, t_1, t_2: hypodermic tubing; *T*, T_1, T_2: plastic tubing.

W is a teflon rod (shown magnified for greater detail in Fig. 3*b*) provided with the two openings 0_1 and 0_2 which serve for withdrawal of separated fractions. These openings communicate through the metal tubes t_1 and t_2 with the plastic tubes T_1 and T_2 which lead to the fraction collectors. The line O_1 O_2 is parallel to the magnet axis. Section S_2 shown in Figure 3*c* is taken through the center of the cell. It shows the iron core *m* and the lucite tubes IT and ET which enclose the annular space AC between them. The teflon insert I (shown in detail in Fig. 3*d*) serves the purpose of introducing the ion mixture to be fractionated. The injection channel I terminating in opening H is aligned parallel to the magnet axis. The metal tube *t* communicates through the plastic tubing T with the reservoir of the solution to be fractionated. Section S_2 shown in Figure 3*c* indicates the placement of the graphite

electrodes E relative to the annular aperture AC of the electrophoretic column. Circular graphite electrodes in the shape of rings surrounding the annular aperture AC are preferable to rod electrodes for mobility measurements to avoid distortion of the ion path, which becomes apparent in the vicinity of the electrode compartments; but for fractionation purposes, the rod electrodes are adequate.

Mode of Operation.—A longitudinal electric current is passed through the annular liquid column in section B by application of a potential difference to the electrodes in the compartments A and C. Due to interaction of this current with the radial component of the magnetic field permeating the annular space AC, the fluid in the annular column is set in rotation. We shall make the initial simplifying assumption that the speed of the fluid is everywhere the same in the annular space. The solution to be fractionated is expelled slowly through the aperture H of insert I by action of gravity from a raised reservoir connected to the hypodermic tubing *t* through a polyethylene tube T (Figs. 3*c* and 3*d*). By choosing a small enough aperture H (0.25 mm), a very fine "streak" of solution is injected into the annular space midway between the walls IT and ET. Due to the longitudinal electric field concomitant with the current passing between the electrode compartments A and C, the ions in the "streak" migrate parallel to the magnet axis with speeds determined by their mobilities and by the potential gradient. The ion path is thus a spiral. The pitch of the spiral is a function of the ion mobility, the longitudinal potential gradient, and the angular velocity of the rotating fluid. Since both the longitudinal velocity of the ions and the angular velocity of the fluid column are proportional to the voltage applied to the cell, the pitch of the spiral ionic path should be independent of the cell voltage, so that voltage fluctuations would not affect the sharpness of the spiral "streaks". This independence has actually been demonstrated experimentally (see below).

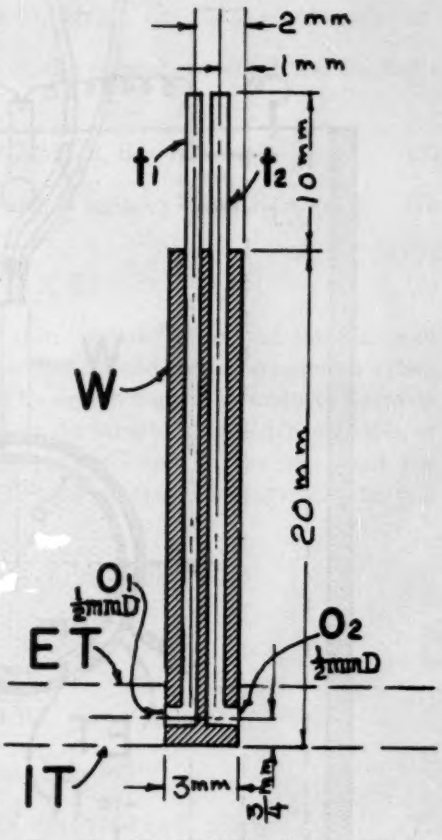


FIG. 3*b*.—*W:* Withdrawal peg. *ET* and *IT:* external and internal boundary, respectively, of the annular electrophoresis channel; O_1, O_2: openings of the withdrawal channels; t_1, t_2: hypodermic tubing.

The approximate magnitude of the pitch to be expected can be calculated as follows taking into account the non-uniformity of the velocity distribution in the annulus. For an annulus whose width is small compared to its mean radius of curvature, the rotary flow of the fluid can be treated, according to Lamb, approximately as a viscous flow between straight parallel plates.[11] Figure 4 shows a trans-

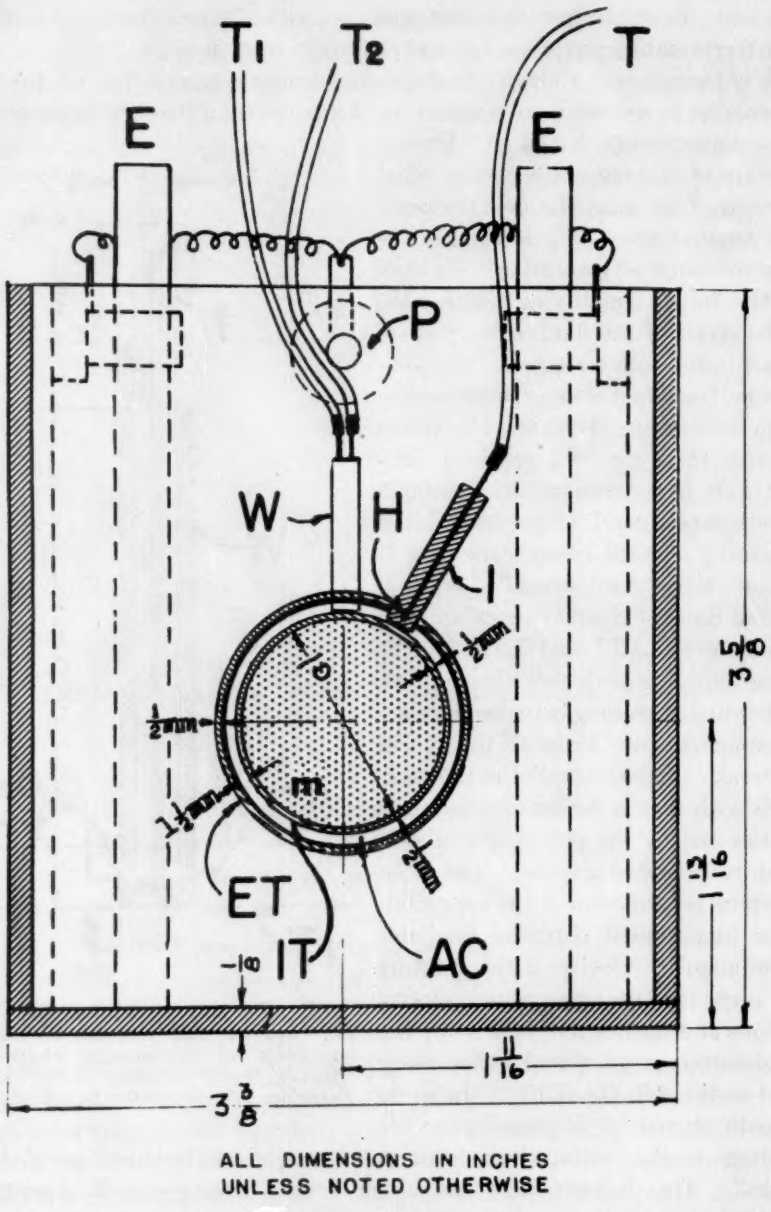


FIG. 3c.—Section through center of the cell. The meaning of *m*, *E*, *P*, *T*, T_1, T_2, *IT*, *ET*, *AC*, *I*, and *W* is the same as in Figs. 2 and 3*a*. *H*: opening through which the solution to be fractionated is injected into the space *AC*.

verse section through the annular tube. The inner radius of the annulus is R and the outer one R + h. The distance of an arbitrary point from the cylinder axis is *r*.

The local tangential velocity u at radial distance r due to rotary motion of the fluid in the annulus is given by

$$u = (1/2\eta)z(z\text{-}h)(dp/dx) \tag{1}$$

where $z = (r - R)$ and dp/dx is the tangential pressure gradient.[11]

The maximum velocity at the center of the annulus is obtained by setting $z = h/2$:

$$u_0 = -(h^2/8\eta)(dp/dx). \tag{2}$$

The value of dp/dx can be determined from the current density $\mathbf{J}$ and magnetic flux density $\mathbf{B}$;

$$-dp/dx = \mathbf{f} = (1/10)[\mathbf{J} \times \mathbf{B}], \tag{3}$$

where $\mathbf{f}$ is given in dynes/cm^3 if $\mathbf{J}$ is measured in amp/cm^2 and $\mathbf{B}$ in gauss. We obtain thus from (2) and (3):

$$u_0 = (h^2/80\eta)[\mathbf{J} \times \mathbf{B}]. \tag{4}$$

Let us now assume that we inject a very thin "streak" of ions at the center of the annulus so that we can confine our consideration to the central maximum velocity u_0. The pitch of the spiral path described by an ion travelling midway between the walls of the annulus is then simply equal to the longitudinal displacement s_0 of the ion during the time required for a complete revolution of the ion about the cylinder axis. The period of revolution τ_0 for the central fluid layer can be calculated simply from (2):

$$\tau_0 = 2\pi/\omega_0 = (1/u_0)2\pi(R + h/2) \cong 2\pi R/u_0. \tag{5}$$

From (4) and (5) follows:

$$\tau_0 = \frac{160\ \eta\pi R}{h^2\ J\ B}. \tag{6}$$

The pitch s_0 is then given by the displacement of the ion:

$$s_0 = v\tau_0 = (d\phi/dy)U\tau_0 = (d\phi/dy)U(160\ \eta\pi R/h^2JB) \tag{7}$$

where the ion velocity $v = (d\phi/dy)U$ is expressed as a product of the ion mobility U and the longitudinal potential gradient $d\phi/dy$. Expressing

$$d\phi/dy = J/\sigma \tag{8}$$

in equation (7) in terms of current density J and the conductivity σ of the solution, we obtain $s_0 = (J/\sigma)U(160\ \eta\pi R)/(h^2/JB)$. Thus,

$$s_0 = 160\ \eta\pi R/h^2\ (U/B\sigma) = (K/B)(U/\sigma). \tag{9}$$

The current density cancels out and the pitch of the spiral ionic path is seen to depend on the constant K, which is determined by the geometry of the cell as well as the viscosity of the fluid, and on the magnetic and electric parameters B, U and σ.

The ion mobility U is thus determined from equation (9) without resort to time measurement:

$$U = B\sigma s_0/K = G\sigma s_0. \qquad (9a)$$

If the factor G = B/K is kept constant, determination of U involves measurement of a conductivity σ and of a displacement s_0. The measurement of U actually amounts to a comparison between the longitudinal velocity acquired by the ions in the electric field $d\phi/dy$ with the tangential velocity imparted to them by the rotation of the fluid column.

The following numerical example illustrates the order of magnitude of the effect. Assume the following data:

$$J = 10^{-2}\ \text{amp/cm}^2;\ h = 0.16\ \text{cm};\ B = 150\ \text{gauss};\ R = 1.5\ \text{cm};$$
$$U = 10^{-4}\ \text{cm/sec/(Volt/cm)};\ d\phi/dy = 50\ \text{Volts/cm};\ \eta = 10^{-2}\ \text{poise}.$$

From (7) follows:

$$s_0 = 50 \cdot 10^{-4}\,(1.6\ \pi\ 1.5)/(2.56 \cdot 10^{-2} \cdot 10^{-2} \cdot 150) \cong 1\ \text{cm}.$$

Effects of the Velocity Distribution and Electro-Osmosis.—In the preceding treatment, the velocity distribution in the rotating fluid was assumed to be parabolic, with the local velocity given by equation (1). This is actually a simplified picture which is correct only in the absence of electro-osmotic streaming. In general, the streaming pattern in the annular fluid column will be complicated by electro-osmotic convection. Due to charge separation at the interface between the electrolyte and the cell wall (ionic double layer) the charged electrolyte layer near the wall is set in motion toward the electrode of opposite polarity. In a closed system, this flow is balanced by a flow in opposite direction at the center of the cell so that the net transfer of fluid through the cell is zero. Figure 5 shows the resulting distribution of longitudinal velocity in a cell of rectangular cross-section whose width h is negligible compared to its depth (measured at right angles to the plane of the paper). This phenomenon has been studied by Smoluchowski,[12] who showed that the velocity distribution in electro-

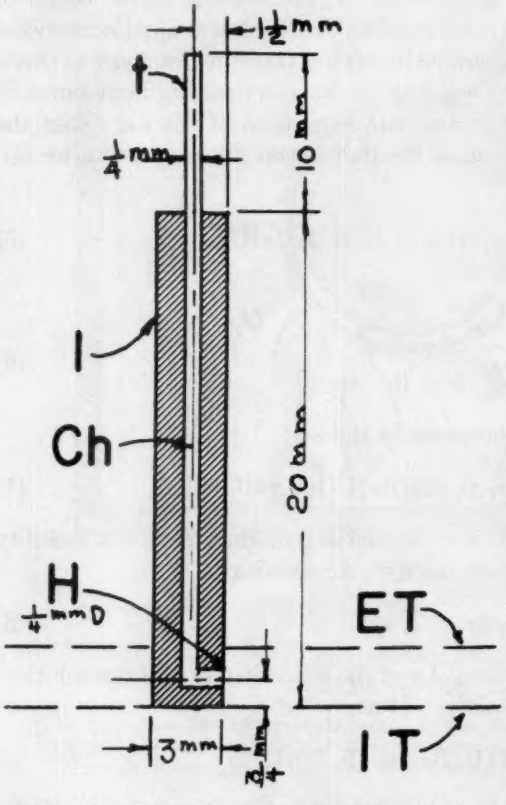


FIG. 3*d*.—I: injection peg; *Ch:* injection channel; *H:* opening through which solution to be fractionated enters the space *AC* bounded by the walls *IT* and *ET;* t: hypodermic tubing.

osmotic streaming is parabolic. The fluid moves in opposite directions at the center and near the cell walls. Two zones (Z_1 and Z_2 of Fig. 5) of zero velocity separate the oppositely directed streams of fluid. The location of these zones is independent of the cell depth h and of the velocity of electro-osmotic streaming.

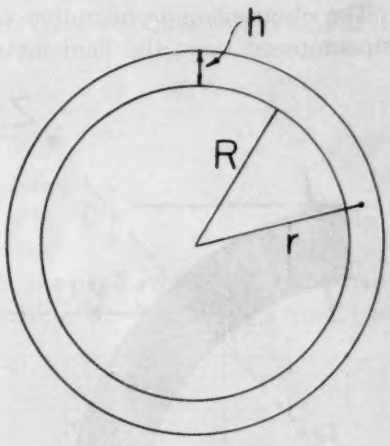


FIG. 4.—See text.

In our annular electrophoresis cell, the electro-osmotic streaming is parallel to the cylinder axis, whereas the electromagnetically engendered circulation is at right angles to it. The velocity distribution is approximately parabolic for both fluid movements. Both, the longitudinal velocity due to electro-osmotic streaming (referred to below for brevity as "longitudinal velocity") and the transverse velocity due to rotation of the annular fluid column (referred to below for brevity as "transverse velocity"), are largest at the center of the annulus and both diminish in parabolic fashion with distance from the center. The fluid elements describe thus a spiral path winding about the axis of the annular cylinder. If both the longitudinal and the transverse velocity distributions were of the form given by equation (1), the pitch of the resulting spiral, which is determined by the ratio between these two velocities, would be constant over the entire width h of the annulus. Actually, as will be shown below, this is not the case. The fluid particles in the zones of zero longitudinal velocity† describe circular paths, whereas the fluid elements beyond these zones move along spirals. If the fluid particles within the space between the cylindrical zones of zero longitudinal velocity describe a right-handed spiral, those outside this space move on left-handed spirals.

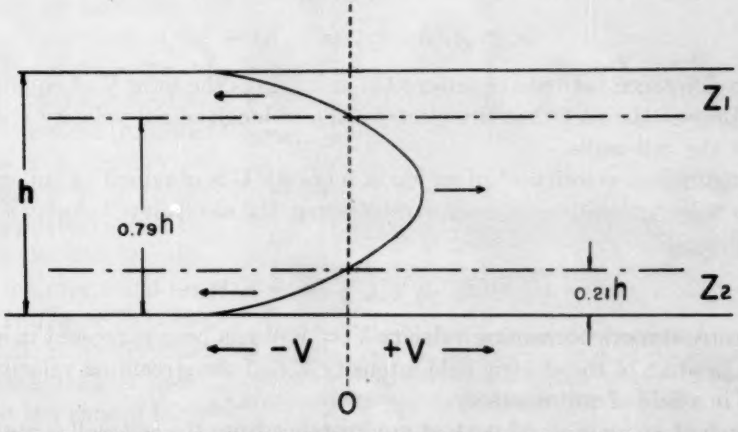


FIG. 5.—Velocity distribution between parallel planes (perpendicular to the page) due to electro-osmotic streaming. The applied electric field is parallel to the arrows. The arrows indicate the direction of flow. Z_1, Z_2: zones of zero velocity (parallel to the walls). h: distance between the parallel planes.

The electrophoretic migration of ions injected into this streaming pattern is superimposed upon the fluid movement caused by the combination of electro-osmotic and electromagnetically engendered convection. The local transverse velocity u of the fluid and the entrained ions is given by equation (1) $u = (1/2\eta)(dp/dx)z(z - h)$. The longitudinal velocity v^* results from two effects: (1) electrophoretic migration and (2) transport due to electro-osmotic streaming.[13] The spatial correlation between the axes u, v, and z is shown in Figure 6. The expression for the electro-osmotic streaming velocity v is very similar to equation (1) giving the transverse velocity u as a function of distance from the cell wall. We obtain v from the differential equation expressing the viscous drag force in terms of the pressure gradient:[13]

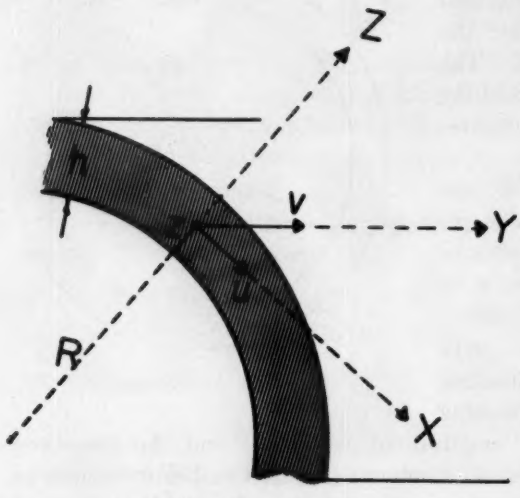


FIG. 6.—See text.

$$dp/dy = \eta(d^2v/dz^2). \tag{10}$$

Integration of equation (10) yields:

$$v = (1/2\eta)z^2(dp/dy) + Az + B. \tag{11}$$

The values of A and B can be found from the boundary condition specifying the magnitude of electro-osmotic streaming velocity V at the walls (i. e., at $z = 0$ and $z = h$). Setting $z = 0$ we get $B = V$. And setting $z = h$ we obtain: $V = (1/2\eta)h^2(dp/dy) + Ah + V$. Thus, $A = -(1/2\eta)h(dp/dy)$ and

$$v = (1/2\eta)(dp/dy)z(z - h) + V. \tag{12}$$

The main difference between equations (1) and (12) is the term V of equation (12) which expresses the fact that the electro-osmotic longitudinal velocity v does not vanish at the cell walls.

The longitudinal velocity v^* of an ion of mobility U is obtained by superposition of the ion velocity relative to the electrolyte upon the electrolyte velocity v relative to the cell:

$$v^* = (1/2\eta)(dp/dy)z(z - h) + E(W - U). \tag{13}$$

The electro-osmotic streaming velocity $V = EW$ has been expressed in equation (13) as a product of the electric field intensity E and the streaming velocity W engendered in a field of unit intensity.

The pitch of an ionic spiral path at any distance from the cell wall is obtained as the longitudinal displacement s during the period of revolution τ of the ion about the magnet axis. Since $\tau \cong 2\pi R/u$ (comp. equation (5)), we get, taking equations (1)

and (13) into consideration and setting u = EC (C being the value of u at unit field strength):

$$s = v^*\tau = 2\pi R v^*/u = 2\pi R\left[\frac{(1/2\eta)(dp/dy)z(z-h)}{(1/2\eta)(dp/dx)z(z-h)} + \frac{E(W-U)}{EC}\right] \quad (14)$$

or

$$s = 2\pi R\left[\frac{dp}{dy}\Big/\frac{dp}{dx} + (W-U)/C\right]. \quad (14a)$$

Due to dependence of C on distance from the wall, the pitch s will be constant over the entire width h of the annulus only for the case W = U. In general, a spiral ion path of sufficiently constant pitch can be obtained for any value of W − U by confining the ions to a narrow zone about the center of the annulus where C varies but slightly in conformity with the parabolic velocity distribution. Accordingly, the experiments described below were performed with thin streaks of ions injected at the center of the annulus.

Experimental Illustrations.—Figure 7 shows spiral streaks obtained with a single ion species India ink. In Figure 7*a*, the non-uniformity of the radial magnetic field near one end of a bar magnet accounts for the variation in the pitch of the spiral. The distribution of the radial magentic field intensity component (recorded by means of a mercury jet magnetometer[10]) is shown above the photograph of the spiral streaks and the diagram of the magnet in Figure 7*a*. In Figure 7*b*, a soft iron cylinder *m* is interposed between the magnet poles. The result is a zone of fairly uniform intensity of the radial magnetic field component, which accounts for the nearly constant pitch of the spiral. The absolute value of the magnetic field was ascertained by means of a mercury jet magnetometer.

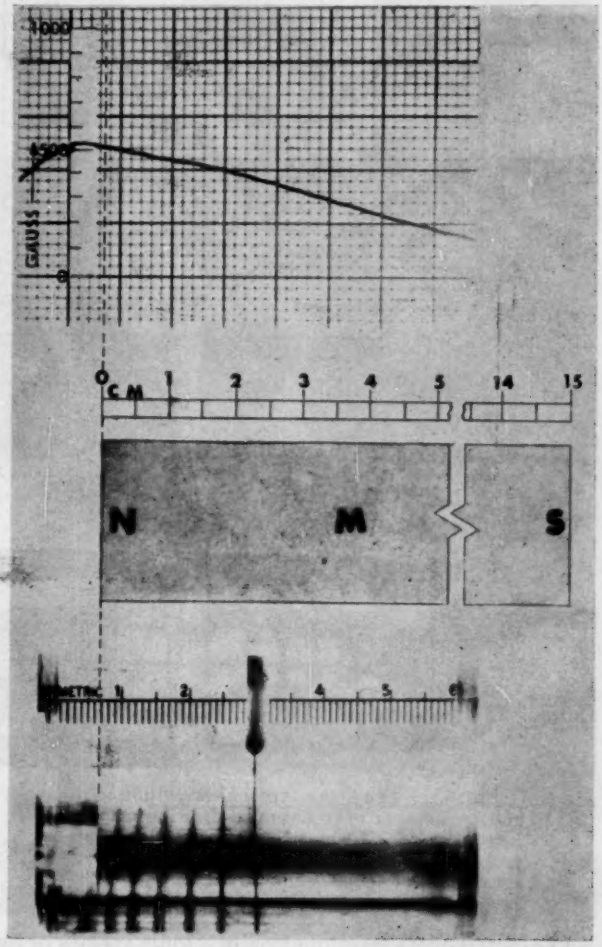


FIG. 7*a*.—Correlation between the magnetic field intensity distribution (top graph) and the pitch of the spiral ion path (bottom photograph) for a single bar magnet (bottom photograph and drawing marked by letters *N*, *M*, *S*).

Figure 8 shows fractionation of the dyes *Evans blue* and *rose bengal* in a dilute pH 5 buffer (obtained by dissolving one pH 5 "Hydrion" buffer tablet in 1 liter of water). After several turns of the spiral, some of the separated fractions may overlap, such as, for instance, *rose bengal* and *Evans blue* in the second streak from the left in Figure 8. The picture is reminiscent of spectral lines of different color obtained with diffraction gratings yielding several orders of interference. The use of several spiral turns is of advantage in separating ions differing but little in mobility.

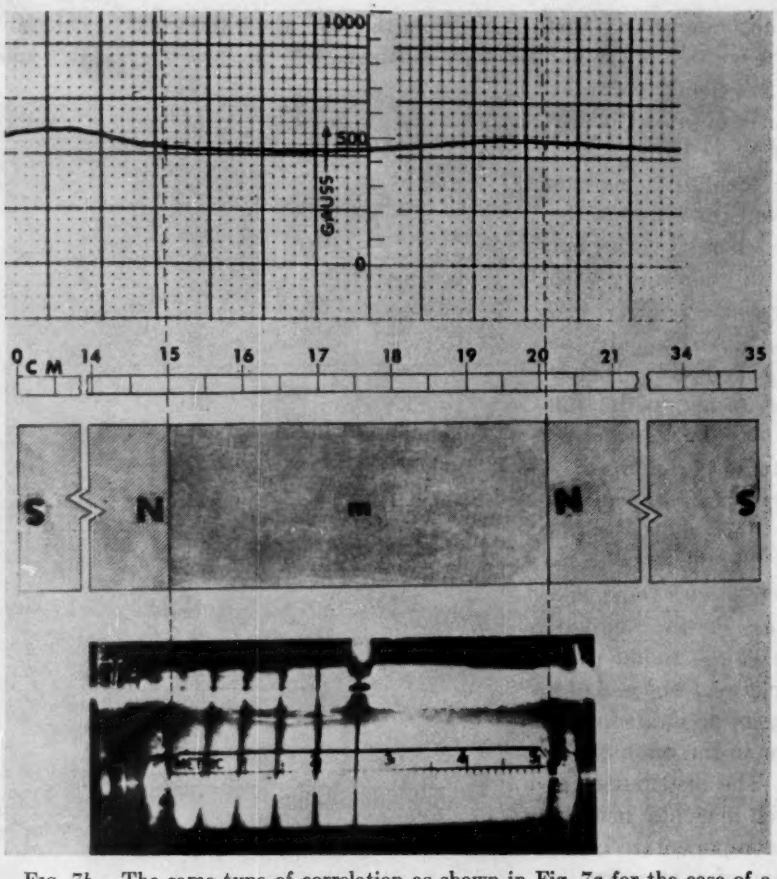


FIG. 7*b*.—The same type of correlation as shown in Fig. 7*a* for the case of a soft iron spacer *m* (see central drawing marked by *S*, *N*, *m*, *N*, *S*) between two like poles (*N*, *N*) of two bar magnets. The magnetic field distribution (top graph) and the distribution of spiral pitch (bottom photograph) are more uniform than for the case shown in Fig. 7*a*.

Figure 9 depicts fractionation of 3 ionic species of like charge using a higher spiral pitch than the preceding fractionation. This separation of a mixture of *India ink*, *rose bengal*, and *Evans blue* is carried out in a dilute pH 7 buffer (obtained by dissolving one pH 7 "Hydrion" buffer tablet in 2 liters of water).

Figure 10 illustrates the effects of variation in voltage across the electrophoresis cell and of variation in the electrical conductivity of the buffer. The spiral of the *Evans blue* ions in Figure 10*a* has been obtained by application of a voltage of 75

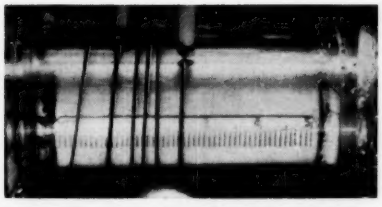


Fig. 8

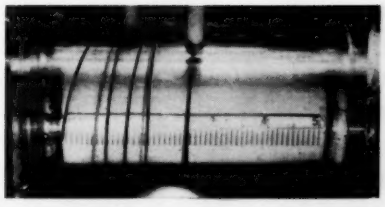
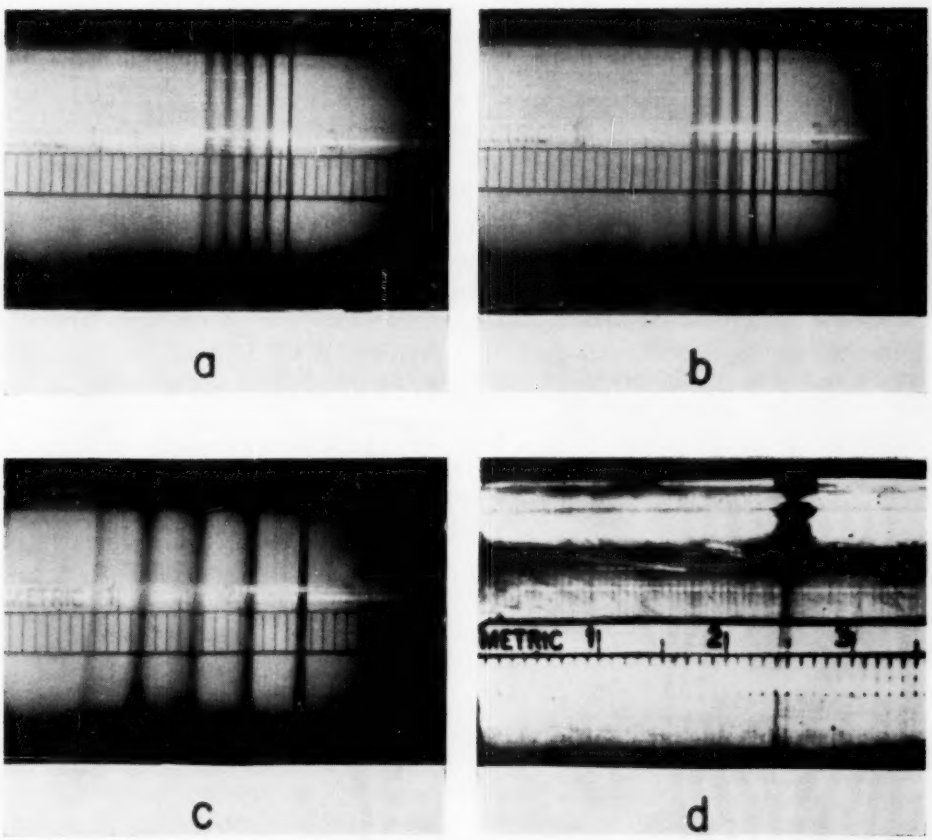


Fig. 9

FIG. 8.—Separation of *rose bengal* from *Evans blue*. The sequence of lines seen from right to left is: (1) initial unresolved streak, (2) rose bengal, (3) Evans blue, (4) rose bengal, (5) overlapping of rose bengal and Evans blue, (6) Evans blue.

FIG. 9.—Separation of a mixture of *India ink, rose bengal,* and *Evans blue*. The sequence of lines seen from right to left is: (1) initial streak in beginning stage of separation, (2) rose bengal, (3) India ink, (4) Evans blue, (5) rose bengal, (6) India ink, (7) Evans blue.



FIGS. 10*a, b, c*.—Effect of cell voltage on the pitch of the spiral. (*a*) Spiral of Evans blue obtained at a cell voltage of 75 volts and cell current of 25 mA. (*b*) Spiral of Evans blue obtained in the same buffer after doubling the cell current and voltage.

Effect of buffer conductivity on the pitch of spiral. (*c*) Spiral of Evans blue obtained at the same current as used in experiment (*a*) after diluting the buffer 1:2.

FIG. 10*d*.—Illustration of separation speed. Separation of rose bengal from Evans blue achieved within approximately 4 sec at a cell voltage of 700 volts.

volts containing pH 7 "Hydrion" buffer (2 buffer tablets per liter of water) at a current of 25 mA. In Figure 10*a* the appearance of the *Evans blue* spiral after doubling the current and voltage is shown. No appreciable change in the pitch of the spiral has been produced.

In Figure 10*c* we see a spiral obtained with the same ionic species in a pH 7 buffer prepared by 1:2 dilution of the buffer used in the experiment illustrated in Figure 10*a* at the same current strength of 25 mA. The pitch of the ionic spiral thus obtained is somewhat more than twice as large as the pitch of the spiral seen in Figure 10*a*.

The speed with which separations of ionic species can be achieved is illustrated by Figure 10*d* which shows a streak containing a mixture of *Evans blue* and *rose bengal* split into two components approximately 4 sec. after injection of the mixture into the fractionation column (pH 5 "Hydrion" buffer; $^1/_2$ buffer tablet per liter of solution; voltage across the cell: 700 Volts).

Having resolved the original mixture of ions into separate components, it is necessary to remove the individual components into separate containers. For adequate resolution, it is desirable to devise a method of withdrawal of separated ion "streaks" which are no more than a few tenths of a millimeter apart. This cannot be accomplished without mixing of the fractions by ordinary methods of pipetting. Trouble-free separation and withdrawal can, however, be obtained by the following device. Figure 11*a* shows the well-known stream line pattern about a cylindrical obstacle obtained by injecting dye on the upstream side of the cylinder. We see that the two stream lines adjacent to the vertical axis of symmetry are spread far apart as they pass the cylinder. This phenomenon can be utilized to separate and withdraw adjacent streaks of different ionic species in our apparatus as shown in Figures 11*b* and 11*c*. Figure 11*b* shows two neighboring streaks of separated ionic species streaming about the cylinder W of Figures 2, 3*a*, 3*b*, and 3*c*. The withdrawal channels O_1 and O_2 of Figure 3*b* (not labeled in Figure 11) are also

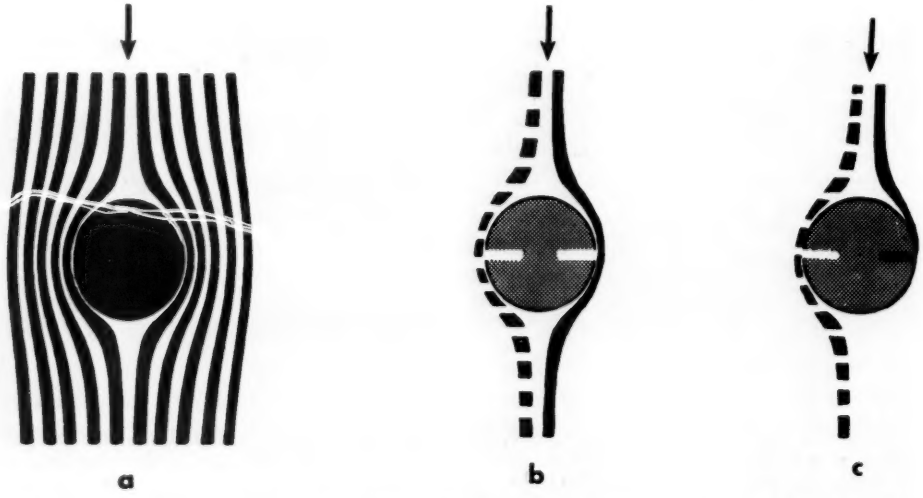


FIG. 11.—Method of separating adjacent streaks. (*a*) stream lines in flow about a cylindrical obstacle, (*b*) separation of adjacent streaks by a cylindrical obstacle, (*c*) withdrawal of a fraction (black streak). The arrows indicate the direction of streaming.

seen in Figure 11*b*. The streaks are removed from each other by the cylindrical obstacle to be withdrawn separately through the diametrically opposed openings O_1 and O_2. Figure 11*c* illustrates the withdrawal of the component shown as a black streak.

The aiming of streaks toward W so as to achieve their separation can be best accomplished by varying the magnetic field intensity. This can be done most simply by longitudinal displacement of the bar magnets. For this reason, it is advantageous not to use a perfectly uniform radial magnetic field, but, rather, an arrangement of magnets providing a section in which B_r varies slowly along the magnet axis.[14]

The method described above is very simple in operation. Its main advantages are: the use of very thin streaks which affords separation of different ionic fractions within a few seconds after traversing of a short migration path, the good stabilization against thermal convection which permits rapid fractionation through use of large potential gradients in cooled electrophoretic columns, the possibility of continuous separation, and the absence of a stabilizing medium, which makes the method suitable for studies of mobilities of suspended particles as well as of ions in solution.

The author is greatly indebted to Drs. B. Cassen and L. B. Robinson for valuable comments, to Messrs. H. S. Tillson and L. Roessner of the Research and Development Shop of the Medical School for help in the construction of the fractionation cells, and to Mr. L. B. Bright for assistance in magnetic measurements and in the preparation of the illustrations.

* This work has been supported by grants from the Office of Naval Research and from the American Cancer Society.

† Zones Z_1 and Z_2 of Figure 5 are, in our annular case, intersections of the plane of the paper with two cylindrical zones of zero velocity.

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[14] As an alternative method of adjustment, *W* can be made to slide in a trough mounted over an axis-parallel slot milled on top of the outer cylinder *ET* of Figure 3*a*.

A NEW HETEROCHROMATIN EFFECT IN DROSOPHILA MELANOGASTER

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The role of heterochromatin in the production in Drosophila of variegated position effects has been thoroughly documented. In brief, variegated phenotypes occur where, as a consequence of chromosome rearrangements, wild-type loci are brought into intimate association with heterochromatic regions. Ostensibly, the heterochromatin of all of the chromosomes is equally effective in inducing variegation. In the following report a new heterochromatin effect will be described in which particular rearrangements of the X-chromosome heterochromatin specifically influence the phenotypic manifestation of a third chromosome mutant.

These experiments stem from a single exceptional female whose phenotype of many extra hairs along the second longitudinal wing vein was not unlike the phenotype associated with the sex-linked Hairy-wing (*Hw*) duplication or with homozygotes for the third chromosome recessive mutant hairy (*h*). This female occurred in an experiment in which males carrying the sex-linked recessive mutant y^2 (yellow-2 body color) were treated with 5500r X-rays and crossed to females homozygous for the X-chromosome scute-8 (sc^8) inversion and the recessive mutants y^2, w^a (white-apricot eye color), *v* (vermilion eye color), and *f* (forked bristles). The exceptional female was crossed to her sc^8 brothers and produced the following progeny: approximately half her daughters had normal wings, half the extra hair phenotype; among her sons approximately three quarter had normal wings, and only one quarter showed the extra hair phenotype. Moreover, those sons showing the extra hair phenotype were without exception sc^8; none carried the irradiated y^2 X chromosome. These results mean that the extra hair phenotype is in all likelihood associated with an autosome and sc^8. In subsequent linkage tests the autosome was identified as the third chromosome. Since the *h* locus, characterized in part by extra wing hairs, is located on the third chromosome, the mutant was tentatively designated hairy-subliminal (h^s). Because h^s homozygotes are usually lethal, a balanced lethal stock $h^s/T(2;3)$Xasta (*Xa*), free of the sc^8 chromosome, was established.

A more detailed examination of h^s individuals, heterozygous or homozygous sc^8, showed that extra hairs are not confined to the wings but may be found in abundance on the head, pleurae, halteres, and occasionally on the scutellum. These are phenotypic characteristics common to homozygous *h* flies.

Linkage tests showed that h^s segregates without exception from h^1, confirming the hypothesis that h^s is localized at the *h* locus. This despite the fact that h^s/h^1, h^s/h^2 and the rare surviving h^s/h^s flies are wild-type in phenotype. However, individuals of each of these genotype, when made heterozygous for sc^8, manifest the hairy phenotype. Since h^s homozygotes are usually lethal, a crossing-over test was carried on to determine whether or not h^s is associated with a structural change in the third chromosome. Crossing over was determined among the progeny of ♀ ♀ h^s/h *gs th* (*gs*=gespleten thorax; *th*=thread arista) with the following results.

For the *h-gs* interval a crossing over frequency of 11.5 per cent was found; for the *gs-th* interval, 7.8 per cent. These results compare favorably with the standard distances of 8.6 per cent and 8.1 per cent, respectively, and demonstrate that no gross chromosomal rearrangement is associated with h^s.

Following the establishment of a h^s/Xa stock, crosses designed to analyze the dependence of h^s on sc^8 for its phenotypic manifestation were carried out. Initially tests were made with the mutant *sc* to determine whether h^s was dependent upon the *sc* effect. In the presence of either heterozygous or homozygous *sc*, h^s is wild-type. Since one break of the sc^8 inversion occurs in the heterochromatin of the X, a number of comparable inversions were employed to determine whether or not they similarly influence the h^s phenotype because of their rearranged heterochromatin. For this purpose ♀ ♀ h^s/Xa were crossed to ♂ ♂ carrying one or the other of eight independent X chromosome inversions all with one break in the proximal heterochromatin. The left wings of 50 F_1 ♀ ♀ $h^s/+$; *inversion*/+ were excised, mounted on slides and counts of the total extra hairs along the second longitudinal wing vein were made.[1] Female progeny from a cross h^s/Xa x Canton-S wild-type ♂ ♂ served as controls. The results of these crosses are included in Table 1 as entries 1–8. Perusal of these results leads to two conclusions: (1) h^s is not dependent solely upon sc^8 for its phenotypic manifestation since similar inversions, sc^{81}, y^{3p} and sc^{L8} (the last as will be demonstrated below) produce comparable effects, (2) not all inversions with breaks in the proximal heterochromatin evince the h^s phenotype.

In addition to the aforementioned inversions, other rearrangements were tested for possible enhancing effect on h^s. These included two inversions not involving the proximal heterochromatin of the X (entries 9 and 10, Table 1), an inversion associated with the heterochromatin of the second chromosome (entry 11), a translocation involving the heterochromatin of the third chromosome (entry 12), and a translocation involving the second and Y chromosomes (entry 13). All were without enhancing effect on h^s. In further tests the amounts of heterochromatin were varied. These included adding an extra Y chromosome to females (entry 14), removing the Y from ♂ ♂ (entry 15) and reducing the heterochromatin of the second chromosome with two deficiences (entries 16 and 17). All failed to evince the h^s phenotype.

By making use of Muller's "left-right" technique of recombining the proximal and distal parts of independent scute inversions, it was possible to decide whether the enhancing effect of the inversions on h^s was casually associated either with the heterochromatin inverted to a new location distal to the centromere region or with the residual, unaltered proximal heterochromatin. The sc^4 and sc^8 inversions will serve as examples for the following predictions. If the enhancing affect on h^s is a function of the displaced heterochromatin of sc^8 the recombinant between sc^8 and sc^4 which includes the left end of sc^8 (including proximal heterochromatin distal to centromere) and the right end of sc^4-designated sc^{8L}, sc^{4R}-should effectively evince the h^s phenotype just as sc^8 does. Alternatively, if the enhancing effect is associated with the unaltered heterochromatin, then sc^{8L}, sc^{4R} should be ineffective and sc^{8R}, sc^{4L} or its equivalent should be effective with h^s. Finally, if the effective factor represents an interaction between both ends of the sc^8 inversions, then neither recombinant with sc^4 need be effective. The recombined inversions in heterozygous condition were tested in $h^s/+$ ♀ ♀. The results of these tests, tabulated in Table

2, clearly demonstrate that in each case where the displaced heterochromatin (the left segment of the effective *sc* inversions) is included in the recombinant chromosome, the phenotypic manifestation of h^s occurs. Entry 4 of this table confirms the conclusion, made above, that sc^{L8} effectively evinces the h^s phenotype.

A further demonstration of the dependence of h^s on particular rearranged heterochromatin came from yet another experiment. As a consequence of sperma-

TABLE 1

INFLUENCE OF HETEROCHROMATIN ON $h^s/+$ PHENOTYPE (PENETRANCE EXPRESSED AS HAIRS ALONG SECOND LONGITUDINAL WING VEIN)

Ancillary Genotype $h^s/+$ ♀♀	Mean number extra hairs* (range)
1. $+/+$	0
2. $sc^8/+$	36 (20–48)
3. $sc^{S1}/+$	19 (5–47)
4. $y^{3p}/+$	6 (0–15)
5. $sc^4/+$	0
6. $w^{m4}/+$	0
7. $rst^3/+$	0
8. $In(1)EN/+$	0
9. $In(1)AM/+$	0
10. $y^4/+$	0
11. $Pm/+$	0
12. $T(1;3)\,w^{v,cb}/+$	0
13. $T(Y;2)C/+$ ♂♂	0
14. XXY	0
15. XO ♂♂	0
16. $M(2)S8/+$	0
17. $M(2)S10/+$	0

* Based on counts of fifty left wings.

TABLE 2

"LEFT-RIGHT" TEST LOCALIZING HETEROCHROMATIN EFFECT OF *sc* INVERSIONS IN $h^s/+$ ♀♀ (0 = NO EXTRA SECOND WING VEIN HAIRS; + = NUMEROUS SECOND WING VEIN HAIRS)

X-chromosome Genetype of $h^s/+$ ♀♀	Presence of extra wing hairs
$sc^{8L}, EN^R/+$	+
$sc^{4L}, sc^{8R}/+$	0
$sc^{S1L}, sc^{4R}/+$	+
$sc^{L8L}, sc^{S1R}/+$	+
$sc^{S1L}, sc^{8R}/+$	+

togonial recombination, exchanges between the distal heterochromatin of sc^8 or sc^{S1} and the Y chromosome have been recovered such that the distal heterochromatin of these inversions is wholly or in part transferred to the Y chromosome.[2] As a logical outgrowth of the aforementioned experiments, it was of importance to test the effectiveness of this heterochromatin in influencing the manifestation of h^s. Four independent transfers of distal heterochromatin from *sc* inversions to the Y chromosome were employed: one recombinant between sc^8 and the Y chromosome; two between sc^{8L}, EN^R and the Y; and one between sc^{S1} and the Y. Each was tested with h^s in ♂♂ and the results tabulated in Table 3. The results establish unequivocally that the distally transposed heterochromatin of sc^8 and sc^{S1} when transferred to the Y chromosome effectively evokes the h^s phenotype. This demonstrates further that the resultant phenotype is a function of the changed location of this heterochromatin and in all probability of an alteration in the heterochromatin itself.

Discussion.—In the foregoing account, eight inversions, each associated with one break in the proximal heterochromatin of the X chromosome, were tested for their effectiveness in conditioning the phenotype of the h^e mutant. Four inversions were found to be effective, four to be ineffective. What distinguishes the two groups? A straightforward basis for understanding the differential effects of the two groups is provided by the timely and detailed cytogenetic analyses of the X chromosome heterochromatin by Cooper.[3] On the basis of a cytological study of the mitotic prophase chromosomes, Cooper delimits the proximal heterochromatin of the X chromosome into the following sequential array of segments and landmarks: a short heterochromatic right arm; the centromere; two blocks of heterochromatin (designated hA and hB by Cooper); the nucleolus organizer region; two blocks of heterochromatin (hC and hD); and the body of the X chromosome euchromatin. As Cooper shows, sc^8, sc^{S1} and sc^{L8} all have their heterochromatic breaks between the centromere and the nucleolus organizer, i.e., in hA and hB, while sc^4, w^{m4} and rst^3 are all associated with breaks in the heterochromatin between the nucleolus organizer and the euchromatin, i.e., hC and hD. The y^{3p} inversion is almost certainly broken in hA since it is described[4] as being slightly longer than sc^8 but, nevertheless, with one break in the heterochromatin to the left of the centromere. All

TABLE 3

EFFECT OF REARRANGED HETEROCHROMATIN LINKED TO THE Y CHROMOSOME ON THE PHENOTYPE OF $h^e/+$ ♂♂ (PHENOTYPE EXPRESSED AS EXTRA HAIRS ALONG SECOND LONGITUDINAL WING VEIN)

Y Chromosome of $h^e/+$ ♂♂	Mean number extra hairs* (range)
Normal Y	0
$sc^8.Y$	38 (26–49)
$sc^{S1}.Y$	23 (11–32)
sc^8, *EN.Y* (1)	31 (11–49)
sc^8, *EN.Y* (2)	34 (17–49)

* Based on counts of fifty left wings.

the evidence points to In(1)EN being associated with a break in the heterochromatic right arm of the X chromosome.[5]

The differential response in evincing the phenotype of h^e can be best understood by assuming first, that the proximal heterochromatin of the X chromosome is neither chemically nor functionally a uniform entity, and second, that as a consequence of breakage and rearrangement a qualitative change in the heterochromatin itself takes place. It means further that not all heterochromatin responds identically to rearrangement, and the facts presented here show that the proximal heterochromatin must be constituted at a minimum of two distinctive types. One type modifies h^e, the other does not. Evidence of a similar nature supporting this conclusion has been alluded to by Demerec[6] who, after studying the cytogenetic nature of a series of *white-mottled* and *Notch* rearrangements in *D. melanogaster*, noted that "the heterochromatin of the X chromosome is not as effective in inducing mottling as the heterochromatin of the autosomes." This despite the fact that in over-all length the X heterochromatin is at least five times greater than that in any of the autosomes. Simultaneously, an even more instructive situation was reported by Demerec. He described two types of Notch mutations associated with X chromosome inversions of the proximal heterochromatin. In two inversions where the

heterochromatin is broken to the left of the nucleolus organizer, mottling of the loci brought into proximity of the nucleolus organizer region was found. In two other inversions where the heterochromatic break occurred to the right of the nucleolus organizer none of the loci show mottling. The euchromatic breaks for all appear to be inseparable. The parallel between these results and those reported here are self-evident. A comparable situation has been recently described by Baker and Spofford[7] whose analysis of the effects of Y chromosome heterochromatin on eye pigmentation of a white-mottled duplication in *D. melanogaster* leads them to conclude that "the heterochromatin of the Y chromosome contains regions which are much more effective than others in influencing the extent of pigmentation." These facts, together with those presented here on h_7, imply that mere rearrangement of heterochromatin cannot explain the various phenotypic effects which accompany such rearrangements. Furthermore, contradictions are encountered when attempting to interpret heterochromatin effects as a function of the amount of heterochromatin subjected to rearrangement. Rather, a more rational explanation follows where it is assumed that a qualitative change in the heterochromatin accompanies rearrangement. Finally, the accumulated evidence supports the thesis that some heterochromatic regions, certainly those of the X chromosome, are not uniform chromosomal elements but to a limited extent, at least, are as variable as euchromatin. Cooper has concluded similarly.[3]

For the moment, one aspect of the situation described here defies a clear-cut explanation. This is the mechanism whereby h^s depends upon the rearranged heterochromatin for its phenotypic effect. Experiments currently under way provide some aid in interpretation. These experiments show that the recessive mutants h^1 and h^2 respond to rearranged heterochromatin as does h^s. Individuals homozygous for h^1 or h^2 and carrying heterozygous sc^8 or sc^{81} manifest extreme hairy phenotypes. A comparable heterochromatin effect does not occur when h^1 or h^2 are heterozygous. From these facts it seems probable that what is involved here is a developmental reaction with a threshold response, with the rearranged heterochromatin effectively providing the means for surpassing the threshold. The precise details are, as yet, not clear. These facts together with the segregation data do, however, confirm the conclusion that h^s is in all probability associated with the *hairy* locus.

Summary.—An allele at the third chromosome *hairy* locus in *Drosophila melanogaster* called h^s is described which depends upon the presence of rearranged X chromosome heterochromatin for its phenotypic manifestation. The cytogenetics of the X chromosome shows that only rearrangements of specific heterochromatic segments influence h^s. These and other facts are interpreted to mean that as a result of rearrangement qualitatively different changes in heterochromatin occur, changes which differentially evoke phenotypic responses.

[1] It is a pleasure to acknowledge the competent, painstaking technical assistance of Ramona and Joachim Eggert. Thanks are also due Professor E. Novitski and Dr. D. L. Lindsley for providing a number of stocks used herein.

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*INHERITANCE OF ANTI-A_1 HEMAGGLUTINATING ACTIVITY IN LIMA BEANS, PHASEOLUS LUNATUS**

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Blood group specific hemagglutinins are essential reagents in certain medical and medico-legal procedures and have found application also in anthropology. Until a few years ago hemagglutinins were obtained solely from animals treated and maintained for that purpose or from human donors.

The discovery that certain plants possess naturally-occurring blood-antigen-specific proteins[1-6] revealed additional potential sources of specific blood grouping reagents. These substances can be extracted from the plant parts which contain them, usually seeds, and used in the same manner as agglutinins from animals; they have the advantage of being much more economical to produce and would be quickly available in case of a large-scale emergency. An extract of *Dolichos biflorus* L is in commercial production as an anti-A_1 agglutinin, and other specific plant agglutinins are known. A brief review of pertinent work on screening plants for agglutinins and a summary of the results of recent studies are presented by Schertz *et al.*[7]

Although inter- and intra-species differences have been observed in agglutinating activity of plants, there is no published information on the genetics of plant hemagglutinins. The purpose of this study was to investigate the inheritance of anti-A, agglutinating activity of lima beans, *Phaseolus lunatus*. In 1945 Boyd[1] observed that some lima bean seeds contained an agglutinin specific for the A antigen of human blood. The large variation in agglutinating activity among varieties of lima beans suggested the possibility that the formation of these hemagglutinins might be gene-controlled. Some lines apparently contained virtually no agglutinin and other lines possessed a high level of agglutinating activity. Lima beans are well suited for inheritance studies because pure lines with genetic markers exist, and an efficient crossing technique is available.[9]

Materials and Methods.—Two lima bean varieties, Fordhook 242 and Cowey, and one genetic stock, L-121, were used as parents in crosses. They were chosen from among 10 lines‡ on the basis of agglutinating activity and genetic markers. Fordhook 242 was selected as the "low" parent because of its low agglutinating activity and a dominant mottling of the primary leaves. Cowey and L-121 were used as parents with high agglutinating activity, the latter having a dominant purple hypocotyl characteristic.

The crossing technique used was that described by Wester and Jorgensen[9] in which stigmas are forced out of large unemasculated flower buds and pollen is deposited on the exposed stigmas. To insure that seed produced by accidental selfing was not mistaken for hydrids, only those seeds which gave rise to plants with the specified markers were used to establish F_2 progenies.

Seeds produced by selfing were harvested from each of the three lines and assayed for agglutinating activity. Parents used in crosses between lines were selected from among those whose selfed progenies showed no segregation. Reciprocal

crosses were made between Fordhook 242 and Cowey and between Fordhook 242 and L-121.

Each seed was cut in half and the portion containing the embryo was planted. The remainder of the seed was divided into two parts and each part was assayed separately for agglutinating activity. The samples were numbered in such a way that the identity of duplicate samples (halves of the same cotyledon) was not known to any personnel of the testing laboratory until all results had been reported. The tests of agglutinating activity were performed in the laboratory of W. C. Boyd in the following manner: The samples were ground and extracted overnight with physiological saline solution in the proportion of 1 gm. of seed to 10 ml of solution. One drop of each extract was tested against one drop of a one per cent suspension of normal red blood cells in physiological saline solution. The extract was tested against A_1, A_2, and B red blood cells. The solution was diluted in steps of 10^1 (1:10) and the highest active (agglutinating) dilution was determined for each sample. The final assay value for each seed was based on the activity of the highest member of the pair of samples for a given seed; when the values did not closely correspond, the data for that seed were discarded (37 cases out of a total of 642). The exponent of the highest active dilution plus 1.5 (no reaction with undiluted equals 0.5) was used in the determination of population means. In view of the method of titers used in determining activity, averages and standard deviations were determined on exponents of 10 rather than absolute dilutions.

Only the results obtained with A_1 erythrocytes are reported here; seeds most active against A_1 were generally active against A_2 also, and sometimes even weakly active with B. It is probable that all these reactions are due to the same agglutinin.[3]

Results.—None of the three lima bean lines used, Fordhook 242, Cowey, and L-121 showed segregation for agglutinating activity and thus were judged to be true breeding for this characteristic. Fordhook 242 had a low activity and differed significantly ($P<0.01$)[8] from the other two lines. Cowey and L-121 had high agglutinating activity and were not found to differ significantly from each other in this respect.

TABLE 1

A_1 AGGLUTINATING ACTIVITY OF PARENTAL LINES AND F_1 PROGENIES FROM RECIPROCAL CROSSES OF "LOW" AND "HIGH" LINES OF LIMA BEANS

Parents and F_1	Number of Seeds Assayed	Mean Highest Active Dilution	
		As Ratio	As Exponent*
Parent			
Fordhook 242	33	1:0.13	$10^{-0.12\pm0.09}$
Cowey	29	1:190	$10^{2.28\pm0.10}$
L-121	27	1:324	$10^{2.51\pm0.07}$
F_1 Cross†			
Fordhook 242 × Cowey	15	1:158	$10^{2.20\pm0.14}$
Cowey × Fordhook 242	7	1:195	$10^{2.29\pm0.17}$
Fordhook 242 × L-121	31	1:182	$10^{2.26\pm0.08}$
L-121 × Fordhook 242	20	1:316	$10^{2.50\pm0.12}$

* ± Standard error of the mean exponent.
† Female parent listed first.

In the cross of Fordhook 242 × Cowey and its reciprocal, 21 F_1s were produced, all of which had a high A_1 agglutinating activity (Table 1). High anti-A_1 activity

was also obtained for each of the 51 seeds resulting from the cross of Fordhook 242 × L-121 and its reciprocal. By mean separation, using standard errors, no statistically significant differences were found between the various F_1 progenies nor between the F_1 progenies and the selfed progenies of the "high" parents.

The F_2 data resulting from assays of 441 seeds are summarized in Table 2. Each of the progenies fits a 3 (high) to 1 (low) ratio with the probability of each of the chi-square values exceeding the 0.01 level.

TABLE 2

A_1 AGGLUTINATING ACTIVITY OF F_2 PROGENIES OBTAINED BY SELFING F_1 OFFSPRING OF RECIPROCAL CROSSES OF "LOW" AND "HIGH" LINES OF LIMA BEANS

	Reaction Category					
	"High"†		"Low"‡		Chi-square, 3 High to 1 Low	Probability Exceeds
F_2 Progeny* from Cross	No.	Per Cent	No.	Per Cent		
Fordhook 242 × Cowey	86	74	31	26	0.14	0.70
Cowey × Fordhook 242	29	88	4	12	2.92	0.05
Fordhook 242 × L-121	155	76	48	24	0.21	0.50
L-121 × Fordhook 242	75	85	13	15	4.91	0.02

* Female parent given first.
† Seeds giving extracts active at dilutions of 1:10 or higher.
‡ Seeds giving inactive extracts or extracts active only when undiluted.

There were differences in the segregation of the progenies of reciprocal crosses. When the low parent, Fordhook 242, was used as the female parent in crosses with each of the high parents, the ratio in the F_2 was nearly a perfect 3:1 as is indicated by the percentages in each reaction category and the low chi-square values (Table 2). However, when Cowey, one of the "high" parents, was used as the male in the crosses with the "low" parent, Fordhook 242, the ratio deviated somewhat from 3:1. An even poorer fit was obtained when the other "high" parent, L-121, was used as the female in crosses with Fordhook 242. The F_2 progenies derived from crosses between a "high" female and a "low" male were thus skewed to the high side.

A re-examination of the F_1 progeny data (Table 1) revealed that the mean progeny values of reciprocal crosses were similarly skewed but the differences, though suggestive, were not significant.

Discussion and Summary.—The inheritance in lima beans of the agglutinin for human erythrocytes of blood group A_1 was investigated by making crosses between a line with low agglutinating activity (Fordhook 242) and two with high activity (Cowey and L-121). The reciprocal crosses also were made. The F_1 progeny all showed high activity, and the F_2's resulting from selfing these segregated approximately in the ratio of 3 "highs" to 1 "low," although the ratios in the F_2 progenies fron the high female by low male cross were skewed to the "high" side.

These results suggest that the agglutinating activity for A_1 erythrocytes is genetically controlled in the three varieties of lima beans tested, but that the genetic ratios among the offspring are also influenced by other factors. The uniformly high activity of the F_1 progeny and the approximate 3:1 segregation in the F_2 progenies indicate that one locus is involved and that the gene for "high" agglutinating activity is dominant.

When the "high" parent was used as the female, the F_2 generation resulting from selfing the F_1's showed an excess of "high" progeny, suggesting an extragenic influence on the ratios. There are a number of possible explanations for the skewness

of the ratio to the high side: (1) Ovule or embryo abortion occurred as a result of an effect of the cytoplasm of the "high" parent. (2) Selective fertilization occurred due to an incompatibility associated with the cytoplasm of the "high" parent. (3) Cytoplasmic inheritance of agglutinating activity was manifested in at least some of the homozygous recessive progeny.

The "low" lines, apparently homozygous recessives, were not completely devoid of activity, and the difference between "high" and "low" lines seems to be merely a difference in the concentration of agglutinin present, or alternatively, in the concentration of one or more agglutinin-inhibiting substances. We have not yet attempted to purify the agglutinin from plants of different activities.

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‡ Appreciation is expressed to R. E. Wester, USDA, Beltsville, Md., and R. W. Allard, University of California, Davis, Calif., for supplying the original seed.

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*THE HUMAN CHROMOSOMAL SATELLITES IN NORMAL PERSONS AND IN TWO PATIENTS WITH MARFAN'S SYNDROME**

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In 1958, quantitative characterizations of all the human chromosomes were published in which measurements of total length and centromere position of each member were presented.[1] These measurements were shown to be sufficiently reproducible to permit unequivocal identification of each chromosome (including the sex chromosomes), provided that a sufficiently large number of clearly defined and well separated mitotic figures is available for analysis, a feature characteristic of the methodology employed by us, since it permits indefinite cell multiplication *in vitro* without change in chromosome constitution.[2, 3] Two pairs of satellited chromosomes were described and characterized, listed as number 18 and 21 in Figure 1. Subsequently two other laboratories also published quantitative morphologic analyses of the entire human karyotype which agreed with ours

virtually completely, within the limits of the experimental uncertainty (which need be no more than a few per cent), and confirmed the group identity of the

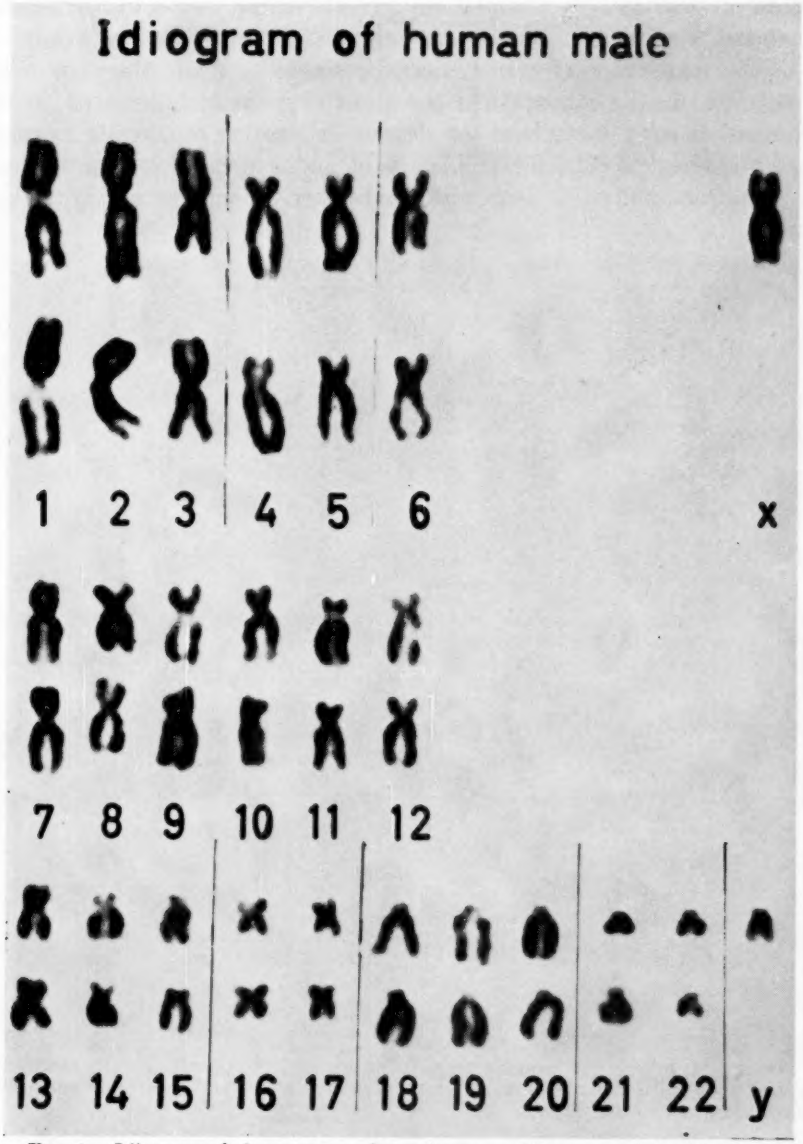

FIG. 1.—Idiogram of chromosomes from the normal human male.[1] The female is identical but with two X chromosomes and no Y. Chromosome pairs number 18 and 21 are satellited as previously described[1] although the structures are so lightly delineated in normal cells that they photograph only faintly and often fail to be reproduced in printing.

satellited chromosomes.[4, 5] However, question was raised whether the satellited chromosome pair of group VII is indeed the largest pair (i.e. number 18 in the Tjio and Puck classification system) or one of the smaller pairs.

In a preceding work, we had described characteristic variations in the satellite structures of two human subjects.[3] These exhibited heterozygously enlarged

satellites, in the one case on both numbers 18 and 21, and in the other only for the latter chromosome. While both of these subjects had been hospital patients, no feature of their records revealed any genetic defect that could be related to their unusual satellite conditions, nor were the patients' families available for study of the inheritance of this cytogenetic marker. While the poor response of the satellites to the conventional chromosome stains and the need to obtain chromosomes in early metaphase for clearest delineation of satellite morphology make such studies difficult, the problem is of an importance warranting careful study. This communication deals with two further findings concerning the human satellites.

FIG. 2.—Typical human chromosome set showing six satellites (arrows). The satellites often are too faint to be well delineated, but this number has now been found in several human subjects, with unrelated genetic conditions.

In analysis of the chromosomes of a variety of human subjects, several instances have been found in which additional satellites are observed. In every case these have been present on another chromosome pair of group VII, probably number 20, in addition to the ones previously described on pairs 18 and 21. The uncertainties of visualization of these structures makes it impossible yet to ascertain whether 6 satellited chromosomes always appear in human cells, but the fact that these are visualized at times in cells from a variety of different human subjects makes it probable that these will turn out to be a frequent, if not universal, characteristic of Man's karyotype. This finding also resolves the apparent discrepancy about the

identity of the satellited pair of group VII, since two pairs can carry these structures. A photograph of a chromosome set with six clear satellites is presented in

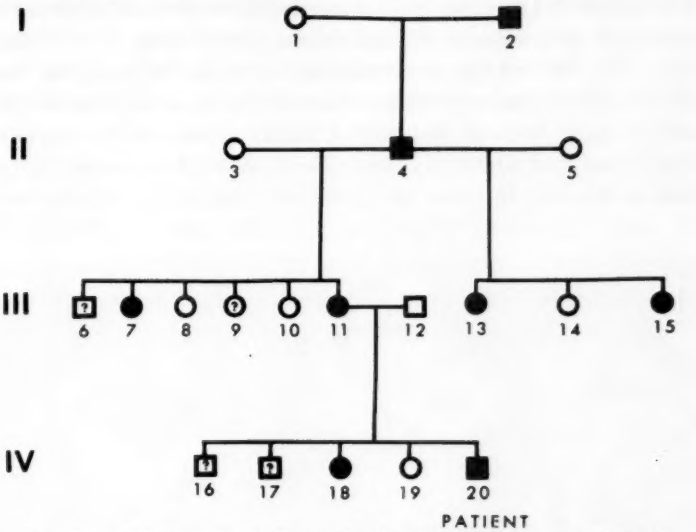

FIG. 3*a*.—Inheritance of Marfan's Syndrome (Patient of Fig. 4). The genealogy was established from the patient's hospital record. Circles indicate females and squares males. Presumptive evidence of the presence of the condition as indicated by heart disease, with or without arachnodactyly and eye defects, has been indicated by a solid figure. Possible presence of the syndrome as indicated by reported death in infancy or other suggestive symptoms is shown by a question mark.

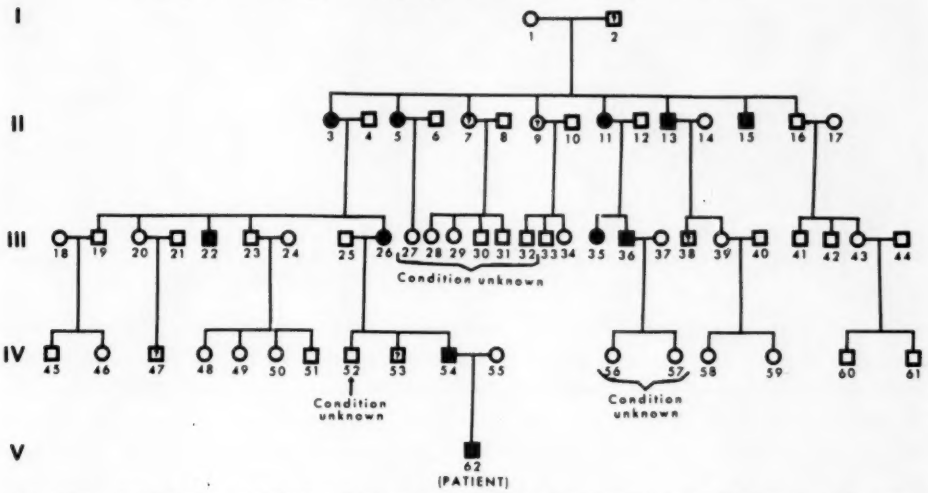

FIG. 3*b*.—Inheritance of Marfan Syndrome in patient corresponding to Figure 5. The genealogy was established from questioning of members of the patient's family, who have been very cooperative. The system followed is the same as in 3*a*. The pattern indicates the typical autosomal, dominant transmission.

Figure 2. It is obvious that means must be found to delineate the satellites with greater reliability.

Further interesting features of the human satellited chromosomes have appeared in an analysis of the cells of patients in whom the disease, Marfan's syn-

drome, had been diagnosed. This condition is a relatively poorly characterized clinical situation, usually inherited as a simple, Mendelian, autosomal, dominant character.[6] However, the first patient available to us had occurred in a family with no history of this disease, or, indeed, any indication of previous metabolic abnormality. The patient was not available for examination by us, and while the diagnosis of Marfan's syndrome was made on the basis of arachnodactyly, congenital heart disease, with probable ventricular septal defect, dislocated lens, a high, arched palate, and arthrogryposis, the absence of any other familial history of this disease renders it uncertain whether this case is identical to those in which a clear, hereditary pattern exists. The chromosomes of this patient were found to be normal, as indicated in a previous publication.[7]

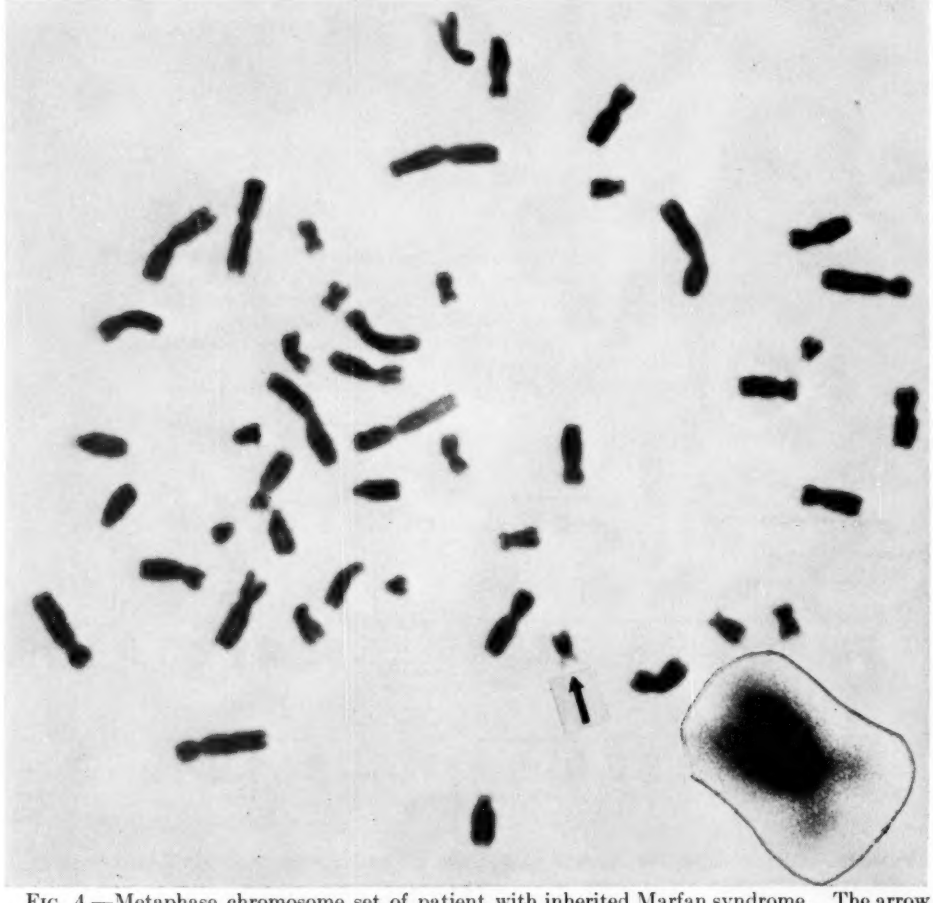

FIG. 4.—Metaphase chromosome set of patient with inherited Marfan syndrome. The arrow indicates the enlarged satellite on one member of chromosome pair 21, which is also shown enlarged in the lower right-hand corner of the picture.

In a second patient in whom the diagnosis of Marfan's syndrome was made, a clear familial history was evident. The pertinent clinical findings were: hypotonia, arachnodactyly, inguinal hernia, pigeon breast, high, arched palate, and cyanotic, congenital heart disease (tricuspid atresia, atrial and ventricular septal defects). The genealogy, indicating transmission by a single, dominant, genetic

determinant is shown in Figure 3*a*. Chromosomal analysis carried out on cells originating from a tiny biopsy of the skin and cultivated *in vitro* in accordance with the methodologies previously described[2, 3, 8] revealed a normal 46/XY complement, with the individual members all essentially normal in total size and arm length. However, an outstanding characteristic of this patient's karyotype is the possession of a tremendously enlarged satellite on one member of pair number 21. This structure, as shown in Figure 4, was consistent in all the cells examined, and was clearly greater than that of any other satellite previously seen, not excluding those which we had already described as enlarged.[3] Unfortunately, no other members of this patient's family are available for study.

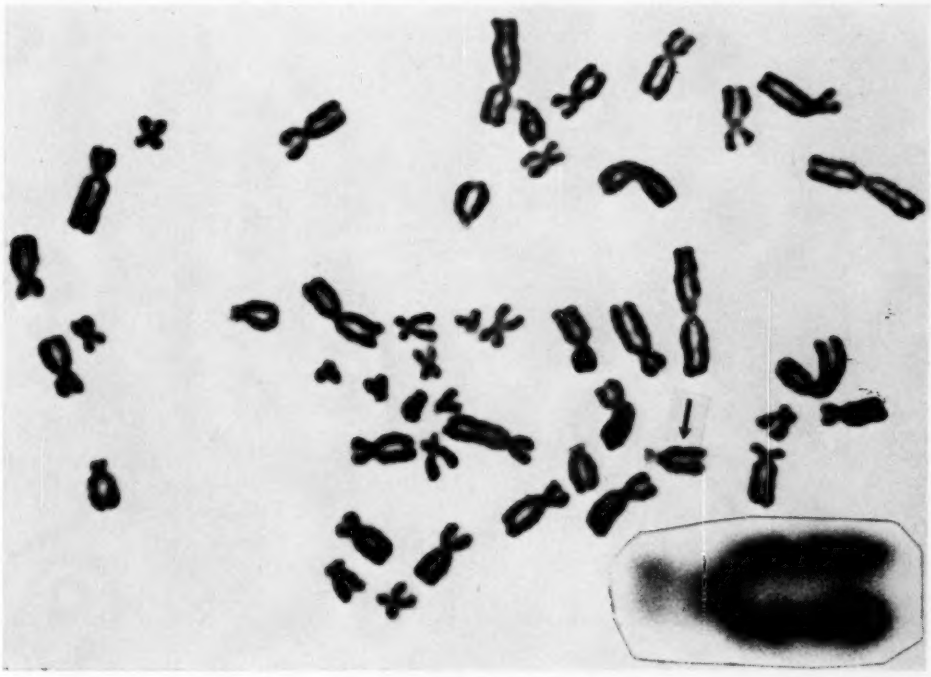

FIG. 5*a*.—Chromosomes of second patient with Marfan syndrome, showing the enlarged satellite on one member of pair 18 (arrow and enlargement in corner).

A third patient with the same diagnosis of Marfan syndrome has also been analyzed chromosomally. The diagnosis was established on the basis of a typical habitus including arachnodactyly, dolichomorphism, marked pectus excavatum and brachycephaly in an 18-month-old boy, and a genealogy indicating a simple, dominant mode of inheritance of Marfan syndrome (Fig. 3*b*). This patient again exhibited a set of chromosomes completely normal except for the satellites (Fig. 5). In this case, however, the abnormality was on a chromosome of pair number 18, instead of 21. Again, it was consistent in all cells, and involved an enlargement clearly beyond the range of those seen in any other human subjects.

Discussion.—The satellite enlargements here described conceivably may arise through any of several mechanisms, such as duplication of the material of which the satellite is composed; interchange or reciprocal translocation between the satellite and part of some other chromosome; or an intrachange within the satellited chro-

mosome itself, such as an inversion containing the satellited region, or a reciprocal interchange between the 2 arms of the chromosome. Hence it is possible that satellite enlargement of itself might be accompanied by a variety of different kinds of phenotypic behavior.

While the present data do not prove the given abnormalities to be the underlying cause of Marfan's syndrome, a relationship is definitely suggested, since

FIG. 5*b*.—An early metaphase chromosome set from the same patient as in 5*a*, demonstrating the enlarged satellite on one member of pair number 18 (lower arrow). Two of the other satellited chromosomes of Group VII are also clearly shown in this picture. Enlargements of each of these chromosomes have been attached close to the corresponding arrows. At the right side an enlargement of one of the satellited chromosomes of pair number 21 has been included for comparison.

in the analysis of chromosomes from more than 30 human subjects, satellites enlarged to the degree here described have been found only in those two patients who exhibit this particular disease, and in the form which displays a dominant genetic pattern. The heterozygosity of the defect in each case is in accord with the mode of inheritance. It is unexpected, however, that the structural defect obtained in these two cases should involve different chromosomes, and a causal connection between this aberration and the given clinical condition can only be maintained on the assumption that damage to either satellite can cause similar

cellular derangement. While the role of satellites in the economy of the cell is still far from clear, evidence shows that the satellited chromosomes involve function of the nucleolus.[9] Therefore, the suggestion is tenable that at least some forms of Marfan's syndrome are due to a dominant genetic defect involving satellite formation which affects a cell function requiring normal interaction of satellites from more than one chromosome. Work is continuing on this problem, investigating satellite cytology and nucleolar behavior in families with this disease. In a forthcoming report, additional pictures will be presented of normal and abnormal human satellited chromosomes, demonstrating their characteristic changes from prophase through late metaphase.

Summary.—Human chromosomal complements have been found with six instead of four satellites. These observations reconcile one of the contradictions in the literature dealing with the human karyotype, and raise the possibility that the higher figure may be a common or possibly universal satellite number in man.

Chromosomes of three patients with a diagnosis of Marfan syndrome have been described. One patient with no familial history of the disease, revealed a normal chromosomal constitution. The other two patients' genealogies exhibited the typical pattern of a simple dominant, autosomal defect. Both patients' chromosomes demonstrated a single, abnormally enlarged satellite, in the one case on a chromosome of pair number 18, and in the other on number 21.

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DIFFERENTIALS AND EXTREMAL LENGTH ON RIEMANN SURFACES*

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1. *Introduction.*—Let $\Gamma_h(W)$ be the Hilbert space of square integrable harmonic differentials on a Riemann surface W.[1] The main theorem, Theorem 1, relates the algebraic structure of $\Gamma_h(W)$ to the geometric structure of W. With this result we are able to give geometric significance to the fact of isomorphism between subspaces of Γ_h for two different surfaces.

The geometric structure of W will be given in terms of the extremal lengths[2] of various homology classes of cycles. If $\{c\}$ is a family of rectifiable curves and ρ is a linear density on W, let $L(\rho, \{c\}) = \inf_{c \in \{c\}} \int_c \rho \, |dz|$ and let $A(\rho) = \iint_W \rho^2 \, dxdy$. Then the extremal length of the family $\{c\}$ is defined to be the supremum of the numbers $L(\rho, \{c\})^2/A(\rho)$ as ρ ranges over all lower semi-continuous linear densities on W.

Let $\Gamma_{he}(W)$ be the exact differentials in $\Gamma_h(W)$ and let $\Gamma_{hse}(W)$ be the semiexact differentials.[3] Let $\Gamma_{ho}(W) = \Gamma_{he}{}^*(W)^\perp$ and let $\Gamma_{hm}(W) = \Gamma_{hse}{}^*(W)^\perp$. If c is a cycle we denote by $\sigma(c)$ the unique real differential in Γ_{ho} such that $\int_c \omega = (\omega, \sigma(c)^*)$ for all $\omega \in \Gamma_h$. The $\sigma(c)$'s are complete in $\Gamma_{ho}(W)$ as c runs through all cycles. We shall denote by $\{c\}$ and $\{c\}_\Omega$ the families of curves in W and Ω respectively which are homologous to c. By $\lambda(c)$ and $\lambda_\Omega(c)$ we shall mean the extremal lengths of $\{c\}$ and $\{c\}_\Omega$.

If W is a bordered surface ∂W will denote the boundary of W. To say that $\omega \in \Gamma_h(W)$ is zero along α, a contour of ∂W, will mean that ω can be extended to be harmonic on $W \cup \alpha$ and that the extension is zero along α.

2. *The Principal Result.*—We list several lemmas that lead up to the main theorem giving one proof.

Lemma 1.[4] $\|\sigma(c)\|^2 \leq \lambda(c)$.

Lemma 2. *Let W be a finite Riemann surface. $\partial W = \alpha \cup \beta$, where α and β are unions of contours of ∂W. Let u be harmonic on $\overline{W}$ such that $u = 1$ on α and $u = 0$ on β. Let c be a dividing cycle of W homologous to α. Then $du = \sigma(c)$.*

Lemma 3. *If W is a closed or finite surface then $\|\sigma(c)\|^2 = \lambda(c)$.*

Proof: We make essential use of the fact that the periods of $\sigma(c)$ over cycles and cross cuts are integers. We define p_1 and p_2, points of $\overline{W}$, to be equivalent if there exists a path γ connecting p_1 to p_2 such that $\int_\gamma \sigma(c)$ is an integer. Since the periods of $\sigma(c)$ are integers $\int_\gamma \sigma(c)$ is an integer for any path γ connecting equivalent points. If E is an equivalence class, then the arc components of E are unions of analytic arcs, over any subarc of which $\sigma(c)$ has zero integral. Locally, then, E is a collection of level curves of a function of which $\sigma(c)$ is the differential. To show that E is closed we assume for $p_n \in E$ that $p_n \to p \notin E$. We can, by passing to a subsequence, join p_n and p_{n+1} by a path l_n so that $0 \neq \int_{l_n} \sigma(c) \to 0$. But this contradicts the fact that $\sigma(c)$ has whole number integrals over paths connecting equivalent points.

Let E_0 be an arbitrary equivalence class if W is closed, and let E_0 be the equiva-

lence class of ∂W if W is finite. It follows that $W - E_0$ is a union of finite surfaces. If R is a component of $W - E_0$ then $\sigma(c)$ restricted to R is exact and is the differential of a function on R of the type considered in Lemma 2. For $\sigma(c)$ restricted to R still has integral periods. If such a period were different from zero we could construct a path starting on ∂R and ending in the interior of R over which $\sigma(c)$ would have an integral of one. This is a contradiction.

Let $W - E_0 = \bigcup_{i=1}^{n} R_i$. $\sigma(c)$ (restricted to R_i) $= du_i$. $\partial R_i = \alpha_i \cup \beta_i$ and $u_i = 1$ on α_i and $u_i = 0$ on β_i. If $\omega \in \Gamma_h(W)$ we have: $(\omega, \sigma(c)^*) = \sum_{i=1}^{n} (\omega, du_i^*) = \sum_{i=1}^{n} \int_{\alpha_i} \omega = (\omega, \sigma(\sum \alpha_i)^*)$. Thus c is homologous to $\sum \alpha_i$. Let c_{is} be the curve in R_i, $u_i = s$. Let $c_s = \sum_i c_{is}$. If ρ is a linear density on W, then using $\sigma(c) + i\sigma(c)^*$ as a local parameter we have:

$$L^2(\rho, \{c\}) \leq \left(\int_{c_s} \rho\sigma(c)^*\right)^2 \leq \int_{c_s} \rho^2\sigma(c)^* \int_{c_s} \sigma(c)^* = \|\sigma(c)\|^2 \int_{c_s} \rho^2\, \sigma(c)^*.$$

Integrating between $s = 0$ and $s = 1$ gives $L^2(\rho, \{c\}) \leq A(\rho)\|\sigma(c)\|^2$ for any ρ. It follows that $\lambda(c) \leq \|\sigma(c)\|^2$. By Lemma 1 we have the desired equality.

LEMMA 4. *Let Ω be an analytically imbedded, relatively compact subset of W containing a cycle c. Consider $\{\Omega\}$, the set of all such Ω, ordered by inclusion. Let $\sigma_\Omega(c)$ denote the period reproducer in $\Gamma_h(\Omega)$. Then $\|\sigma_\Omega(c)\|_\Omega \to \|\sigma(c)\|$ as $\Omega \to W$.*

LEMMA 5. *If $\Omega \subset W$ then $\lambda_\Omega(c) \geqq \lambda(c)$.*

THEOREM 1. *If W is an arbitrary surface $\|\sigma(c)\|^2 = \lambda(c)$.*

Proof: Using the previous lemmas we have:

$$\lambda(c) \geqq \|\sigma(c)\|^2 = \lim_{\Omega} \|\sigma_\Omega(c)\|_\Omega^2 = \lim_{\Omega} \lambda_\Omega(c) \geqq \lambda(c).$$

COROLLARY 1. $\lambda_\Omega(c) \to \lambda(c)$.

COROLLARY 2. *Let W be a bordered surface; α and β two disjoint contours of ∂W. Let c be a curve connecting α to β, and let $\{c\}$ be the family of curves weakly homologous to c. Let $\sigma(c)$ reproduce the integral along c of harmonic differentials that vanish along α and β. Then $\|\sigma(c)\|^2 = \lambda(c)$.*

Proof: Double W along α and β, and then apply Theorem 1.

3. *Applications.*—THEOREM 2: *Let W and W' be homeomorphic surfaces. Let c and c' be corresponding cycles. Then the following two conditions are equivalent:*

(1) *$\exists M \geqslant 1$ such that $M^{-1}\lambda(c) \leq \lambda(c') \leq M\lambda(c)$;*

(2) *$\exists \phi$, a continuous linear isomorphism from $\Gamma_{ho}(W)$ onto $\Gamma_{ho}(W')$ such that $\phi\sigma(c) = \sigma(c')$ and $\int_c \sigma = \int_{c'} \phi\sigma$ for all c, c'.*

Proof: Theorem 1 makes it obvious that (1) follows from (2). For the converse we first observe that linear combinations of the $\sigma(c)$'s with real rational coefficients are dense in the subspace of real differentials of Γ_{ho}. Using a common denominator for the coefficients we see that elements of the form $m^{-1}\sum_{k=1}^{p} n_k\sigma(c_k) = m^{-1}\,\sigma(\sum_{k=1}^{p} n_k c_k)$ are dense; that is, the real multiples of the $\sigma(c)$'s are dense. Via Theorem 1 the inequality of (1) can be extended to this dense subset. Thus a linear map ϕ of the type considered in (2) is seen to be uniformly continuous on a dense subset of the real differentials in $\Gamma_{ho}(W)$ and the range of ϕ is a similar subset of $\Gamma_{ho}(W')$. (2) now follows easily.

COROLLARY 3. $(\omega, \sigma^*) = (\phi\omega, (\phi\sigma)^*)$.

Definition: Two surfaces satisfying the conditions of Theorem 2 will be called *ho-equivalent.*

COROLLARY 4. Quasiconformally equivalent surfaces are *ho*-equivalent.

A simple example shows that the converse of Corollary 4 is false.

If $W \in O_{HD}$ then $\Gamma_{ho}(W) = \Gamma_h(W)$ and so $\Gamma_{ho}(W) = \Gamma_{ho}{}^*(W)$. This observation leads to the following theorem.

THEOREM 3. *If W and W' are homeomorphic surfaces of class O_{HD} then the following three conditions are equivalent:*

(1) W and W' are ho-equivalent;

(2) $\exists M \geqslant 1$ such that $\lambda(c') \leq M\lambda(c)$ for all c and c';

(3) $\exists \phi$, a linear map from $\Gamma_h(W)$ into $\Gamma_h(W')$ such that $\int_c \sigma = \int_{c'} \phi\sigma$.

Proof: (3) implies (2): By the closed graph theorem ϕ is continuous. (2) implies (1): Since $\Gamma_h = \Gamma_h{}^*$,

$$\|\omega\| = \max_{\sigma} \frac{|(\omega, \sigma^*)|}{\|\sigma\|}.$$

Since the real multiples of the $\sigma(c)$'s are dense in the real differentials and since $(\sigma(c), \sigma(c_1)^*)$ is the intersection number, $c \times c_1$, of c and c_1, we have:

$$\lambda(c_1) = \|\sigma(c_1)\|^2 = \max_{c} \frac{(\sigma(c_1), \sigma(c)^*)^2}{\|\sigma(c)\|^2} = \max_{c} \frac{(c_1 \times c)^2}{\lambda(c)} \leq$$

$$M \max_{c'} \frac{(c_1' \times c')^2}{\lambda(c')} = M\lambda(c_1').$$

THEOREM 4. *Two closed surfaces, W and W', of the same genus are conformally equivalent if and only if $\lambda(c) = \lambda(c')$ for corresponding cycles.*

A property that holds simultaneously on two *ho*-equivalent surfaces will be called *ho*-invariant. The most immediate *ho*-invariant properties are related to the structure of Γ_{ho}. In stating properties that depend on a particular canonical homology basis, the *ho*-invariance must, of course, be referred to corresponding canonical homology bases on the two surfaces involved. We list several *ho*-invariants:

(1) The validity of the generalized bilinear relation.[5]

(2) Generalizations of the bilinear relation of the type considered by Kusunoki for parabolic surfaces.[6]

(3) $\Gamma_{ho}(W) \cap \Gamma_{he}(W) = \Gamma_{hm}(W)$.

(4) For surfaces of class O_{HD} that analytic differentials are determined by their A-periods.

The proofs of these facts follow from Corollary 3.

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HOMOLOGY AND HOMOTOPY OF H-SPACES

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It is a classical result of E. Cartan,[1] which follows also from recent results of Bott,[2] that if G is a Lie group then $\pi_2(G) = 0$. Hopf[3] showed, by a strictly algebraic argument, that the first nonvanishing rational cohomology group of a finite dimensional H-space is in an odd dimension, which implies that the first homotopy group which has a free summand must be in an odd dimension. His argument rested on the notion of a diagonal map in an algebra. An algebra with such a map is now known as a Hopf algebra.

In this note we state some results in the homology theory of H-spaces, and as a consequence obtain a theorem on homotopy which includes Cartan's theorem as a special case. A full treatment will appear elsewhere.

Our method is to study the torsion in the homology of a space using the following construction communicated to the author by J. C. Moore.[4]

Let C be a free chain complex over the integers i.e., $C = \sum_i C_i$, $\partial: C_i \to C_{i-1}$ and $\partial^2 = 0$. Let p be a prime, and consider the exact sequence $0 \to Z \to Z \to Z_p \to 0$. If we tensor it with the chain complex C we get an exact sequence of chain complexes $0 \longrightarrow C \xrightarrow{\alpha} C \xrightarrow{\beta} C \otimes Z_p \longrightarrow 0$ and thus in homology we get an exact couple

$$\begin{array}{ccc} H(C) & \xrightarrow{\alpha_*} & H(C) \\ {\scriptstyle \partial_p}\nwarrow & & \swarrow{\scriptstyle \beta_*} \\ & H(C \otimes Z_p) & \end{array}$$

Moore has remarked that the spectral sequence of this couple has

$$E^\infty \cong \beta_* \text{ (Free part of } H(C))$$

and furthermore $d^{(1)} = \beta_* \partial_p = \beta_1$ is the Bockstein boundary associated with the exact coefficient sequence $0 \to Z_p \to Z_{p^2} \to Z_p \to 0$. The higher order differentials $d^{(r)}$ may be interpreted as higher order Bocksteins.

Let X be a topological space whose homology groups are finitely generated in each dimension (all spaces henceforth will be assumed to have this property).

If we take C to be the singular chain complex of X, the above construction yields the "Bockstein spectral sequence" of X in homology mod p, and similarly taking

C to be the cochain complex we get the "Bockstein spectral sequence" in cohomology. This cohomology spectral sequence of X is made into a ring by using the diagonal map $\Delta: X \to X \times X$, $\Delta(x) = (x, x)$. If X is an H-space, i.e., there is a continuous map $\mu: X \times X \to X$ and a point $e \in X$ such that $\mu(x, e) = \mu(e, x) = x$ for all $x \in X$, then μ induces a ring structure in the homology spectral sequence. The two maps Δ and μ together induce Hopf algebra structures[5] in the homology and cohomology spectral sequences of X, and the two Hopf algebras are dual to each other.

Let A_* be a Hopf algebra over Z_p such that A_i is a finite Z_p-module for all i, A^* its dual Hopf algebra.

Definition. An element $x \in A_m$ is said to have ∞*-implications* if there exists a sequence $x_0, x_1, \ldots, x_n, \ldots$, with $x_0 = x$, $x_i \in A_{mp^i}$, $x_i \neq 0$ for all i, such that for each $i > 0$ there exist integers f, j with $f > 0$, $0 \leq j < i$ such that either

(1) $x_i = x_j^{p^f}$ or

(2) there exist elements $\bar{x}_i, \bar{x}_j \in A^*$ such that $\bar{x}_i(x_i) \neq 0$ and $\bar{x}_j(x_j) \neq 0$ with $\bar{x}_i = \bar{x}_j^{p^f}$.

(If A_* or A^* is nonassociative we define by induction $z^n = z^{n-1} \cdot z$.)

Clearly the existence of an element $x \in A_m$ of ∞-implications implies that $A_{mk} \neq 0$ for all k.

An element $x \in A_*$ is said to be primitive if, letting $\Delta: A_* \to A_* \otimes A_*$ denote the diagonal map in the Hopf algebra A_*,

$$\Delta x = x \otimes 1 + 1 \otimes x.$$

THEOREM 1. *Let X be an arcwise connected H-space, and let $\{E^{(r)}\}$ be the Bockstein spectral sequence in homology mod p for X. If $y \in E_{2n+1}^{(r)}$, y is primitive, and if $d^{(r)}y = x \neq 0$, then x has ∞-implications in $E^{(r)}$.*

In the proof of Theorem 1, the duality between $E^{(r)}$ and $E_{(r)}$ (the cohomology spectral sequence) is exploited, together with some properties of the Bockstein spectral sequences for the spaces $K(Z_{p^r}, 2n)$.

As a consequence we have

THEOREM 2. *If X is an arcwise connected H-space and there exists an integer N such that $H_m(X; Z_p) = 0$ for $m > N$, then the first nonvanishing group $\pi_q(X) \otimes Z_p$, for $q > 1$ occurs for q odd. In particular, $\pi_2(X) \otimes Z_p = 0$.*

Applying Theorem 2 for all p we get:

THEOREM 3. *If X is an arcwise connected H-space and there exists an N such that $H_m(X; Z) = 0$ for $m > N$, then the first nonvanishing higher homotopy group $\pi_q(X)$, $q > 1$, occurs for q odd. In particular, $\pi_2(X) = 0$.*

This theorem includes the theorem of E. Cartan mentioned above, since any Lie group G satisfies the hypothesis (with N = dimension of G).

The proofs are in the spirit of Hopf's result mentioned above, being mainly algebraic, and only at one point does geometry enter through the study of $K(Z_{p^r}, 2n)$.

Among other applications of the Bockstein spectral sequences for an H-space is the following:

THEOREM 4. (*Poincaré Duality Theorem.*) *Let X be an arcwise connected H-space, and suppose $H_m(X; Z) = 0$ for $m > n$ and $H_n(X; Z) \neq 0$. Then*

(1) $H_n(X; Z) = Z$

(2) $H_{n-1}(X; Z)$ *is free*

(3) *if* ν *is a generator of* $H_3(X; Z)$ *then cap product with* ν $\cap \nu: H^q(X; Z) \to H_{n-q}(X; Z)$ *is an isomorphism for all* q.

In this sense, finite dimensional H-spaces are homologically like manifolds. There is apparently no known example of a finite dimensional H-space which is not the homotopy type of a manifold.

* The author is a National Science Foundation Postdoctoral Fellow.

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*ON THE USE OF ABSTRACT ALGEBRA AS A METHOD FOR OBTAINING RESULTS INVOLVING RATIONAL INTEGERS ONLY AND THE REVERSE OF THIS PROCEDURE**

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In the subject of number theory, of course, many parts of mathematics have been employed in deriving theorems which involve in their statements rational integers only. We may divide, rather roughly, these contributions under the general headings of modern algebra, geometry, and analysis.[1] Conversely, different number theoretic problems and patterns have led mathematicians to new results in many parts of pure and applied[2] mathematics.[3–5]

In the present paper we shall be concerned mainly with the applications of modern algebra to number theory, particularly the number theory of rational integers as well as the results obtained in modern algebra which seem to have been suggested by arithmetic patterns. (An eminent example is the formulation of the concept of *rings*, which seems likely to have originated from the study by leading mathematicians of the properties of the set of algebraic integers in an algebraic field.) Under the heading of "modern algebra" we have included the classical theory of algebraic numbers, but we shall pay more attention to those parts of present-day algebra which are not regarded as belonging to said theory, such as semigroup and group theory, finite rings and fields, matrices and other noncommutative algebras, etc. As to our title, more than half the papers the writer has written on number theory, including algebraic numbers, contain in them results of this character. Obviously, then, such subjects are very close to him.

Up to approximately ten years ago, it seems that if we exclude elementary number theory, then by far the greater portion of articles published under the heading of number theory belong to analytic number theory. However, it seems that within the last ten years the proportion of articles based on advanced algebraic methods has been gradually increasing. We shall not discuss results here, however, the proofs of which use both analysis and modern algebra, or analysis alone.

The present paper constitutes only a beginning in our investigation of the relations between abstract algebra and number theory, and we have not as yet attempted to locate all the literature bearing on this topic. We hope eventually to publish more papers along this line.

Our principal aim in pursuing the present investigation is to isolate, if possible, certain tools in abstract algebra or the theory of algebraic numbers which seem to have been the most successful in deriving results concerning the rational integers alone. Comparison of the articles where such methods have been used for this purpose should then, perhaps, lead to important new ideas and results.

In our selection of results described in what follows, we probably give a bit more space to the statements of what seem to be curious or unusual relations rather than to the discussion of methods and theorems which are rather well known. In line with this, for example, we do not give much attention to the parts of number theory in which the development depends mainly on the use of linear transformation. We shall, however, try to give something of the historical background of all the subjects treated.

It will be convenient to begin our more detailed discussion by setting up certain distinctions. We shall define elementary algebra and elementary number theory as these two subjects were known prior to about 1770, and so it seems it would be proper to start with Lagrange[6] in our discussion of the development of algebra and its application to number theory, as he appears to have been the first to employ linear substitutions in connection with algebraic or arithmetic forms. As to the latter, he investigated binary quadratic forms, using this tool in a systematic manner, and obtained important results.

Gauss made fundamental contributions to number theory by the use of algebraic methods other than elementary. This he did in particular in Chapter V in his *Disquisitiones Arithmeticae*, 1801, by developing the ideas of linear substitutions in connection with binary and ternary quadratic forms. Further, in Chapter VII, using a theory of the algebraic solution of the equation $x^n = 1$, given by Lagrange[7] (the theory now usually known by the name *cyclotomy* (Kreistheilung)), he obtained the various arithmetical and geometrical results particularly pertaining to the construction of a regular polygon with 17 sides. He also obtained curious and remarkable relations between trinomial congruences, binomial coefficients, and certain binary quadratic forms. One of these[8] is as follows: If p is a prime in the form $4n + 1$ and $p = x^2 + y^2$,

$$x \equiv \frac{1}{2}\frac{2n!}{(n!)^2}, \bmod p; \; x \equiv 1, \bmod 4, \tag{1}$$

and a somewhat similar explicit form for the residue of y, modulo p. This result is extended by others, including Stickelberger,[9] and Hasse-Davenport.[10]

In the famous memoirs of Gauss[11] on biquadratic reciprocity, he made the first advances toward what we now call the classical theory of algebraic numbers by defining an integer in the field generated by i with $i^2 = -1$ as an expression of the form $a + bi$ with a and b rational integers. He then stated, without proof, the law of biquadratic reciprocity, which involves the use of these complex numbers. The relation of this theorem to the theory of rational integers may be described as follows.

In the theory of rational integers, for example, we are enabled, using an algorithm based on the law of quadratic reciprocity, to find the integers x, if any exist, such that $x^2 \equiv a \pmod{p}$ with p prime. Similarly, by the use of an algorithm based on biquadratic reciprocity, we can determine the integers such that $y^4 \equiv b \pmod{p}$. In the above congruences, x and y and a and b are rational integers. Eisenstein[12] followed the work of Gauss by defining integers in a field generated by a primitive cube root of unity. He obtained a theorem of cubic reciprocity which provides an algorithm for discovering if an integer z with $z^3 \equiv c \pmod{p}$, where z and c are rational integers, exists. In his proof of the law of cubic reciprocity Eisenstein used formulas from the subject of cyclotomy, which we have already mentioned. Following these investigations, many applications of formulas in cyclotomy, in order to obtain results involving rational integers only, were made by Jacobi, Dirichlet, Cauchy, and others, and some of these will now be discussed. For example, Dirichlet[13] showed how solutions in rational integers $y(\neq 0)$ and x may always be obtained for the Pellian equation, $x^2 - ny^2 = 1$, where n is a rational integer > 1, making use of some results in cyclotomy.

Jacobi[14] and Cauchy[15] obtained the relation (closely related to equation (1))

$$x^2 + \Delta y^2 = p^k,$$

where $k = (\Sigma b - \Sigma a)/\Delta$, $p = \Delta n + 1$, and Δ is an integer of the form $8m + 7$. Also, Σa stands for the sum of the quadratic residues a of Δ, $0 < a < \Delta$, and Σb is the sum of the quadratic nonresidues b, $0 < b < \Delta$. If Δ is a prime number in the above, the expression

$$\frac{\Sigma b - \Sigma a}{\Delta}$$

is the number of improperly primitive classes of quadratic forms of determinant $-\Delta$. This result was conjectured by Jacobi in advance of Dirichlet's proof that the statement is true. The Jacobi-Cauchy result above was derived by the use of cyclotomy, and the method was entirely algebraic and different from that employed by Dirichlet in deriving a closed form for the class number of any binary quadratic homogeneous form. The latter method involved the use of analytic expressions for the class number.

If l is an odd prime, Kummer used a primitive lth root of unity, say ζ, and defined integers in the field generated by ζ as being of the form

$$a_0 + a_1\zeta + \ldots a_{l-2}\zeta^{l-2},$$

where the a's are rational integers. He developed a theory of such expression which is generally called the theory of cyclotomic fields. He made these investi-

gations in order to be able to generalize the cubic law of reciprocity to the case where we have lth powers instead of cubes. Also, he wished to make applications to Fermat's last theorem.[16] In efforts to prove this statement, he built up a theory of ideals in such cyclotomic fields. In his work along those lines he found it necessary to use analytic methods in addition to algebraic ones.

So far we have discussed how efforts to solve problems in number theory have led to the founding of the theory of linear substitutions, the theory of certain types of algebraic numbers, and the various developments in cyclotomy. We now come to the next important step in which efforts to solve an arithmetic problem led to important new developments in the theory of matrices. In 1860 H. J. S. Smith[17] attacked a problem of finding criteria that a system of linear congruences, modulo m, with m an arbitrary integer, has solutions, the coefficients in the congruences being rational integers also. We shall not state what these conditions are, but his result depends on what is sometimes called *Smith's normal form* a of matrix, namely, that every matrix A of rank r with elements in a principal ideal ring R, is equivalent to the diagonal matrix $[h_1, h_2, \ldots, h_r, 0, \ldots, 0]$, where $h_i | h_{i+1}$. Smith employs this in the case where R is the ring of rational integers, and A is a square matrix. *It seems probable that this was the first time the concepts of elementary divisors and invariants of a matrix appeared in the mathematical literature.*

Dedekind and Kronecker later extended Kummer's notions in order to find the general classical theory of algebraic numbers, namely, the consideration of those fields and rings defined by means of an equation of the nth degree in one unknown with rational coefficients. This gave mathematicians more tools for obtaining results involving rational integers only.

H. Weber,[18] in his *Algebra*, seems to have been the first to employ to some extent the theory of abstract Abelian groups to number theory. He called the residue classes modulo m "Zahlklassen." Hilbert,[19] in his *Zahlbericht*, refers to a ring of algebraic numbers, this term being employed as we use it today. The concept was used independently by Kronecker and Dedekind under different names.

Around the turn of the century the development of abstract algebra proceeded rapidly, particularly through the work of E. H. Moore, Dickson, Wedderburn, and Steinitz. Moore[20] defined an abstract finite field and proved that any such finite field could be represented by means of a Galois finite field. Dickson[21] made many contributions to this theory. He also developed a theory of linear groups in which the coefficients of the linear forms used were elements in a finite field instead of the field of residue classes, modulo p, as had been employed by C. Jordan. Wedderburn[22] made many important contributions to abstract algebra, in particular to the algebra of matrices. E. Steinitz[23] developed the theory of fields in general.

In 1909 Thue obtained his celebrated theorem that if

$$a_n x^n + a_{n-1} x^{n-1} y + \ldots + a_1 x y^{n-1} + a_0 y^n = c, \qquad (2)$$

with c and the a's given integers and $a_n \neq 0$, $n > 2$, then equation (2) has only a finite number of integral solutions. The proof was obtained by the use of properties of algebraic numbers.

In 1912, H. S. Vandiver[24] developed a theory of finite rings, said theory having been suggested by generalizations of the theory of residue classes modulo m where m is any integer, the latter theory being included in the former.

L. J. Mordell[25] used algebraic numbers and quaternions in a number of valuable papers he has written on Diophantine analysis.

In 1938, James Singer proved the following theorem:[26] "A sufficient condition that there exist $m + 1$ integers, $d_0, d_1, \ldots, d_m$, having the property that their $m^2 + m$ differences $d_i - d_j$, $i \neq j$; $i - 0, 1, \ldots, m$, are congruent, modulo $m^2 + m + 1$, to the integers $1, 2, \ldots, m^2 + m$, in some order, is that m be a power of a prime." The proof depended on the idea of the use of the concepts of finite projective geometry and finite fields. The writer does not know of any proof using rational integers only. Singer also gave a generalization of the theorem.[27] These results, it seems to the writer, exhibit a remarkable relation between the additive and the multiplicative properties of integers, and they have received considerable attention from a number of mathematicians.

A. Hurwitz[28] applied quaternions in order to prove a number of theorems concerning the number of representations of positive integers as the sum of four integral squares.

Dickson[29] developed arithmetics of various types of algebras, particularly those that were then called hypercomplex numbers and made application to various Diophantine equations.[30]

Now consider the theory of substitutions in group theory. If we write the substitution

$$S = \begin{pmatrix} 0 & 1 & 2 & 3 & 4 \\ 0 & 1 & 3 & 2 & 4 \end{pmatrix}$$

we note that as x ranges over the complete set of residue modulo 5, x^3 gives a rearrangement of the x's given in S. Under these circumstances S is said to be an analytic representation[31] of the substitution. This subject has been investigated by Hermite, Dickson, L. J. Rogers, and others.[32] Dickson[33] considered linear forms with variables $x_1 \ldots x_m$ with indetermine integral coefficients modulo p, a prime. He then defined invariants and covariants involving such forms and later considered similar forms where the integral coefficients are replaced by elements of finite fields. He made many contributions to this theory as well as to related theories.

Following Eisenstein[34] there is considerable literature on the application of linear transformation to the theory of representing an integer in the form

$$f = (a, b, c, d) = ax^3 + 3bx^2y + 3cxy^2 + dy^3$$

where a, b, c, and d are rational integers. It was investigated by Eisenstein, Arndt, Pepin, and others.

The little-known theory of Hermitian forms has also been applied to number theory. Following Hermite[35] we write

$$v = x + iy, \qquad v_0 = x - iy,$$

and u_0 for the conjugate imaginary of u, and we have the binary Hermitian form

$$f(v, u;\ v_0, u_0) = Avv_0 + Bvu_0 + B_0 v_0 u + Cuu_0,$$

where A and C are real, while B, B_0 are conjugate imaginaries. Then f takes

only real values under a linear transformation and $BB_0 - AC$ is an invariant, called the determinant of f. Definite, indefinite, and reduced forms are defined, and a theory has been built up which is analogous to that of binary quadratic forms. The subject has applications to the theory of quaternary quadratic forms and has been developed mainly by Hermite, Picard, Bianchi, and Humbert. (Cf. Dickson.[36])

Vandiver[37] (1947) used some results published in his 1912 paper[24] on finite algebras (rings) in order to develop concepts and results in the theory of Abelian groups.

In 1922 Hecke published his well-known *Theorie der Algebraschen Zahlen*. Here he seems to have been the first to have employed the theory of groups both finite and infinite in setting up a fairly coherent elementary theory of the arithmetic of the rational integers. He also employed some group theoretic ideas in his chapters on algebraic numbers. H. Hasse[38] proceeded further along these lines in utilizing Abelian group theory in a development of elementary number theory and the elements of the theory of algebraic numbers. Vandiver and Milo W. Weaver[39] carried some of these ideas further by introducing the elements of the theory of semigroups in setting up a part of elementary number theory.

The Diophantine equation

$$dx^2 + cy^2 = z^m, \qquad dc = n\ (d > 1, c > \mathrm{q}),$$

has been examined by a number of mathematicians, employing the theory of quadratic fields, and expressions have been found for all the solutions of it provided in the case where the class number of the quadratic field defined by the $\sqrt{-n}$ is prime to m where m is odd. What appear to be the principal papers on this topic are by Pepin,[40] A. Brauer,[41] and M. Ward.[42]

Criteria that the integer 2 is an eth power residue of a rational prime has been considered for small values of e by several writers. Beginning with Gauss, who proved that 2 is a biquadratic residue of a prime p of the form $4n + 1$ if and only if p is representable as $x^2 + 64y^2$, Reusche[43] stated that criterion for the octavic character of 2 modulo $p = 8n + 1$. This was proved by A. E. Western.[44] A. Cunningham[45] discovered the criterion for the 16th power residue of the prime p of the form $16n + 1$, which was later proved by A. Aigner,[46] who used the class field theory in his proof. A. L. Whiteman,[47] employing simpler methods depending on cyclotomy, obtained another proof of the 8th and 16th power residue characters of 2. The method coincides with that used by Gauss[48] for the proof of the biquadratic character of 2 when it is modified to suit this case. All the criteria just mentioned depend on the possibility of representing p as some quadratic form.

A. A. Albert[49] proved that the set of positive ternary forms with integral coefficients, of determinant $d = \gamma^2\delta$, with δ square-free, will represent all and only those positive integers a which are not in the form $a = \rho^2\sigma$, σ square-free, $\sigma = \alpha\delta$, $\alpha = 8n + 7$, $(\alpha, d) = 1$ and the Jacobi symbol $(p/\alpha) = 1$, for all prime factors p of d. This result is an elegant generalization of the well-known result that all positive integers which are not of the form $4^k(8n + 7)$ can be expressed as the sum of three squares.

Latin squares are closely related to finite projective geometries and finite fields, so E. T. Parker[50] employed the latter to advantage in some of his work on Latin squares.

At the present time it is not always the results recently discovered which are applied to obtain new theorems. The tools used may be quite old. For example, Emma Lehmer[51] employed a result in cyclotomy obtained by Kummer in 1846 in order to obtain her new results concerning cubic and quartic residues of rational odd primes. These results give direct criteria that certain rational integers are quartic or cubic residues modulo p. These theorems were derived independently of the use of the laws of cubic and quartic reciprocity.

Eckford Cohen,[52] in a number of recent papers, has developed properties of Abelian groups which were obtained by the study of the behavior of arithmetic functions. He also has reversed this process and applied the theory of Abelian groups to obtaining properties of arithmetic functions.

Many articles have been written treating the exponential sums used in cyclotomy in order to obtain results concerning conditional equations in finite fields, which usually give new results concerning rational integers, since the finite field of order p^n, p prime, is when $n = 1$, isomorphic to the field of residue classes modulo p. L. Carlitz, L. K. Hua, H. H. Mitchell, Vandiver, Whiteman, and others have contributed to these topics.[53]

As we indicated quite early in this paper, our work here is only beginning. However, I should like, in closing, to indicate what we should be concerned with in future articles which we hope to publish on the same general topic. We recall first that we are confining ourselves in the present paper to the consideration of results involving rational integers only, or results which lead to them. However, we have noted in the past the number of published papers in which theorems regarding algebraic numbers were obtained. Yet they might have been specialized to give new theorems concerning the rational integers only. Few of the authors of these papers signalized this fact, however, and in connection with the present paper we have not gone into this type of investigation.

At the beginning of this paper we stated that we would not consider any of the literature which involved algebraic as well as analytic methods in connection with a particular problem in number theory. We did this in order to avoid the necessity of a detailed examination here of proofs of this character, in order to compare the part of each proof depending on analysis and the part depending on algebra. Of course if we did make an examination of this kind in connection with a particular theorem, it may turn out that the use of modern algebra in the proof might be of great importance and therefore worth discussing in a future paper.

Of course when we use algebraic numbers in order to work out new results concerning rational integers, we usually are employing algebraic extensions of the original rational ring or field. Further, in the case where we employ, as has been done in some investigations, the law of reciprocity between lth powers in a cyclotomic field defined by lth roots of unity, l being prime, we are going further, as no proof of this law of reciprocity is known which does not involve the use of results in the theory of Kummer fields, which are, of course, extensions of cyclotomic fields.

In view of the above statements, I think it quite likely that methods depending on other types of algebraic extensions will be very useful in obtaining new results in number theory involving rational integers only. As we glimpse in that direction, it is noted that Iwasawa[54] obtained as a special case of certain theorems derived by

means of a method just mentioned, relations involving class numbers of fields defined by l^n-th roots of unity, $n \geqq 1$. In fact he finds

$$e_n = kn + ml^n + c,$$

where e_n is the highest power of l dividing the class number of C_{n+1}, which denotes the cyclotomic field of order l^n-th, l a prime $\neq 2$, where $k = k(L/K)$, $m = m(L/K)$ are defined by a certain extension of C_n, and c is a suitable integer independent of n. This result connects up with the known properties of C_{n+1}, namely that there exists a closed explicit form of the value of this class number,[55] and the result[56] derived from this gave criteria that the first factor of said class number be divisible by l. It is possible that this result may be extended to the case where the modulus is l^n, $n > 1$ by using similar methods. Proof of this last statement depends on what appear to be entirely different considerations than those involved in the proof of Iwasawa's statement. Often when this sort of thing turns up in mathematics the results are exceptionally interesting, particularly when, after further study, relations between the methods are discerned.

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[1] The late J. F. Ritt used to tell us that he regarded number theory as "applied mathematics" because so many different parts of mathematics has been applied to it.

[2] In this connection we shall refer to Harvey Cohn, "Some Applied Number Theory," *J. Soc. Indust. Appl. Math.*, **4**, 152–167 (1956). See also A. Brauer, "A Problem of Additive Number Theory and Its Application in Electrical Engineering," *J. Elisha Mitchell Scientific Soc.*, **61**, nos. 1 and 2 (1945).

[3] Such applications have been at least to the theories of semigroups, groups, finite rings and fields, and semirings.

[4] We have an extensive branch of number theory called the "geometry of numbers," and of course numerous applications have been made to number theoretic problems which are not always regarded as belonging to the geometry of numbers, but geometric methods are used. Here we shall cite such a problem,[26] as considerable literature has grown up around it. The first mention of a special case of it seems to have been by O. Veblen (*Am. Math. Monthly*, **13**, 46 (1906), reprinted by Johnson Reprint Corp., New York,) who proposed it to the readers of the *Monthly*. He stated the result for $m^2 + m + 1 = 43$. Cf. Singer[26] for definition of m.

[5] Since we are not going to discuss in detail the application of number theory to analysis or the reverse, we shall refer the reader to Hilbert in his introduction to his *Zahlbericht* of 1897 (*Werke*, bd. 1, 63–68), who discussed the applications of the theories of algebraic numbers to analysis as well as the applications of analysis to the theory of algebraic numbers, which had been made prior to 1897. Of course, if any mathematician treated in detail the advances which have been made along these lines since, the work would be immense. It would include also applications of abstract algebra, included in the theory of algebraic numbers, to analysis and then conversely.

[6] "Recherches d'arithmetique," *Nouv. Mem. Acad. Berlin*, 265–312 (1773); *Oeuvres*, **3**, 693–758.

[7] Lagrange (*Abh, Acad. Berlin*, for years 1770 and 1771) introduced the "Lagrange Resolvent"; Gauss, *Disquisitiones Arithmeticae*, Sec. 7.

[8] Gauss, "Theoria residuorum biquadraticorum, commentatio prima," *Werke* **2**, Art. 23. This is a very unusual type of relation in number theory since binomial coefficients and related arithmetic functions are usually very complicated from the standpoint of divisibility.

[9] Stickelberger, L., "Uber eine Verallgemeinerung der Kreistheilung," *Math. Ann.*, **37**, 321 (1890).

[10] Haase-Davenport, *J. Reine Angew, Math.*, **172**, 151–182 (1935).

[11] "Theoria residuorum biquadraticorum, commentatio prima et secunda," *Werke*, **2**, 65–93.

[12] "Beweis des Reziprozitätsgesetzes für die kubischen Reste in der Theorie der aus dritten Wurzeln der Einheit zusammengesetzten komplexen Zahlen," *J. für Math.*, **27** (1844). "Démonstration du théorème fondamental sur les résidus biquadratiques qui comprend comme cas particulier le théorème fondamental," *J. für Math.*, **28** (1844).

[13] "Sur la maniere de resoudre l'equation $t^2 - pu^2 = 1$ au moyen des fonctions circulaires," *Werke*, **1**, 343.

[14] *J. für die Mathematik*, **9**, 189 (1826).

[15] *Oeuvres* (1), nearly all of vol. **3**.

[16] The applications of the theory of algebraic numbers to Fermat's last theorem and closely related equations are so well-known that they will not be discussed in this paper.

[17] *Philosophical Trans.*, **151**, 293–326 (1861); *Proc. London Math. Soc.*, **4**, 236–253 (1873).

[18] Second edition, Braunschweig, 1899. Galois considered a prime p and a polynomial in an indeterminate x of degree n with given integral coefficients, say $F(x)$, which is also irreducible modulo p. He then spoke of a "root" of this congruence. If α is one such, then the set,

$$a + a_1\alpha^2 + a_2\alpha + \ldots + a_{n-1}\alpha^{n-1}$$

with the a's ranging over 0, 1, . . ., $p - 1$, independently, give p^n distinct elements. Weber (p. 306) notes that such elements form a *finite field* of order p^n.

[19] *Werke*, **1**, 121 (1932).

[20] Math. Papers Read at the International Math. Congress, 1893, published by the Am. Math. Soc., New York, 210 (1896).

[21] *Linear Groups*, B. J. Teubner, Leipzig (1901).

[22] Cf. references to Wedderburn on page 204 of A. A. Albert's *Structure of Algebras* (New York: Am. Math. Soc., 1939).

[23] *Algebraische Theorie der Körper*, originally published in *Crelle* (1910), reprinted by Chelsea Pub. Co., New York (1950).

[24] "Theory of Finite Algebras," *Trans. Math. Soc.*, **13**, 293–304 (1912).

[25] Cf. for example, "On the Integer Solutions of the Equation $ey^2 = ax^3 + bx^2 + cx + d$," *Proc. London Math. Soc.*, **21**, Part 6, 415–419 (1922); "The Integer Solutions of the Equation $y^2 = ax^n + bx^{n-1} + \ldots + k$," *J. London Math. Soc.*, **1**, Part 2, 66–68 (1926); "An Application of Quaternions to the Representation of a Binary Quadratic Form as a Sum of Four Linear Squares," *Quarterly J. Math.*, **8**, 58–61 (1937).

[26] *Trans. Am. Math. Soc.*, **43**, 379–381 (1938). Cf. also our footnote 4.

[27] *Ibid.*, 385.

[28] Göttingen Nachrichten, 311–340 (1896). Amplified in his *Vorlesungen über die Zahlentheorie der Quaternionen*, Berlin (1919).

[29] *Algebras and Their Arithmetics* (The University of Chicago Press, 1923).

[30] *Ibid.*, 198.

[31] *History of the Theory of Numbers III*, (Carnegie Institution of Washington, D. C., 1923) Chap. 23.

[32] *Ibid.*, 290–291.

[33] *Ibid.*, chap. 19.

[34] *Ibid.*, chap. 12.

[35] *Ibid.*, 269.

[36] *Ibid.*, chap. 15.

[37] "On a p-Adic Representation of Rings and Abelian Groups," *Ann. Math.*, **48**, 22–28 (1947).

[38] *Vorlesungen über Zahlentheorie*, Springer-Verlag, Berlin (1950); *Zahlentheorie*, Akademie-Verlag, Berlin (1949).

[39] "Introduction to Arithmetic Factorization and Congruences from the Standpoint of Abstract Algebra," *Am. Math. Monthly*, **65**, No. 7 of the Herbert Ellsworth Slaught Memorial Papers, 1–53 (1958).

[40] "Sur certains nombres complexes de la forme $a + b\sqrt{-c}$," *J. Math.*, 3^e serie, Bd 1, 317–372 (1875).

[41] "Über einige spezielle diophantische Gleichungen," *Mathematische Zeitschrift*, Verlag-Springer, Berlin, 499–504 (1926).

[42] "The Diophantine Equation $X^2 - DY^2 = Z^M$," *Trans. Am. Math. Soc.*, **38**, 447–457 (1935).

[43] "Mathematische Abhandlung, enthaltend neue Zahlentheoretische Tabellen," Programm zum Schlusse des Schuljahrs 1855–56 am Königlichen Gymnasium zu Stuttgart (1856).

[44] "Some Criteria for the Residues of Eighth and Other Powers," *Proc. London Math. Soc.* (2), **9**, 244–272 (1911).

[45] "On 2 as a 16-ic Residue," *Proc. London Math. Soc.* (1), **27**, 85–122 (1895).

[46] "Kriterion zum 8. and 16. Potenzcharacter der Reste 2 und -2," *Deutsche Math.*, **4**, 44–52 (1939).

[47] "The Sixteenth Power Residue Character of 2," *Can. J. Math.*, **6**, 364–373 (1954).

[48] *Werke*, **2**, 67–92 (1876).

[49] Albert, A. A., "The Integers Represented by Sets of Ternary Quadratic Forms," *Am. J. Math.*, **55**, 274–292 (1933).

[50] "Orthogonal Latin Squares," these PROCEEDINGS, **45**, 859–862 (1959).

[51] *Mathematika*, **5**, 20–29 (1958).

[52] *Notices of the Am. Math. Soc.*, **6**, 278–9, 513 (1959).

[53] There is an extensive bibliography of articles published along this line in A. A. Albert's *Fundamental Concepts of Higher Algebra* (Chicago: Univ. of Chicago Press, 1956), pp. 152–155.

[54] "On $\perp$-Extensions of Algebraic Number Fields," *Bull. Am. Math. Soc.*, **65**, 183–226 (1959). Iwasawa's formula for e_n, given above, yields the result that for $n = 1$; $e_n = 0$, provided e is regular.

[55] Jacob Westlund, "On the Class Number of the Cyclotomic Number Field," *Trans. Am. Math. Soc.*, 201–212 (1903).

[56] Vandiver, H. S., "On the Class Number of the field $\Omega(e^{2i\pi/p^n})$ and the Second Case of Fermat's Last Theorem," these PROCEEDINGS, **6**, 416–421 (1920); "On the First Factor of the Class Number of a Cyclotomic Field," *Bull. Am. Math. Soc.*, **25**, 458–461 (1919).

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*HOMOLOGY THEORIES AND DUALITY**

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1. It has been known for some years that the integral cohomology group $H^n(X; \Pi)$ of a (reasonable) space X with coefficients in an abelian group Π can be characterized as the group of homotopy classes of maps of X into a certain space $K(\Pi,n)$. The spaces $K(\Pi,n)(n = 0, 1, 2, \ldots)$ are related by the fact that each is the loop-space of the next; and it is this property that is crucial in setting up the apparatus of cohomology theory. In fact it is known that, if $\mathbf{E} = \{E_n\}$ is a sequence of spaces, each the loop-space of the next, then the groups $H^n(X; \mathbf{E}) = [X; E_n]$ satisfy all of the Eilenberg-Steenrod axioms for a (reduced) cohomology theory except the dimension axiom.

It is natural to ask whether there are corresponding *homology* theories. Of course, if a cohomology theory is given one can define homology groups (at least for a finite complex X) as the cohomology groups, suitably reindexed, of the complement of X in a sphere of sufficiently large dimension. This definition is awkward to work with and has the disadvantage of failing to be intrinsic. The object of this note is to develop an intrinsic theory of generalized homology groups, and to prove

an Alexander duality theorem, as well as a Poincaré duality theorem for a suitable class of manifolds. A full account will be published elsewhere.

2. A *spectrum*[1] $\mathbf{E}$ is a sequence $\{E_n \mid n \in Z\}$ of spaces with base point, together with a sequence of maps $f_n\colon E_n \to \Omega E_{n+1}$. The spectrum $\mathbf{E}$ is said to be an Ω-spectrum if and only if each map f_n is a weak homotopy equivalence. A *convergent* spectrum is one with the property that, for some N, E_{N+k} is k-connected for all $k \geqq 0$.

If $\mathbf{E}$ is a spectrum, X a base with base point, the reduced points $X \times E_n$ naturally form a spectrum $X \times \mathbf{E}$. The n-th homotopy group $\pi_n(E)$ of a spectrum $\mathbf{E}$ is the direct limit of the sequence $\pi_{n+k}(E_k)$.

If X is a space with base point, and $\mathbf{E}$ a spectrum, we define reduced cohomology and homology groups of X with respect to $\mathbf{E}$ by

$$\tilde{H}^n(X; \mathbf{E}) = \varinjlim [S^k X, E_{n+k}]$$

$$\tilde{H}_n(X; \mathbf{E}) = \pi_n(X \times \mathbf{E})$$

The corresponding non-reduced groups of a space X (without base-point) are defined to be the reduced groups of the space X^+ obtained from X by adjoining a point P, the base point of X^+. With suitable definitions of relative groups and connecting homomorphisms δ^*, ∂_*, we have

THEOREM 1. *The homology and cohomology theories with respect to a spectrum* $\mathbf{E}$ *satisfy the Eilenberg-Steenrod axioms, except for the dimension axiom, on the category of compact polyhedra.*

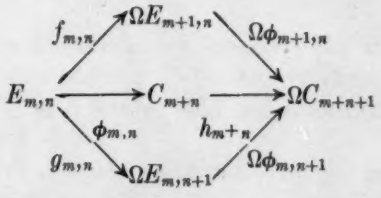
Clearly $\tilde{H}^n(S^0; \mathbf{E}) \approx \pi_{-n}(\mathbf{E})$, $\tilde{H}_n(S^0; \mathbf{E}) \approx \pi_n(\mathbf{E})$. Hence, if $\mathbf{E}$ is an abelian group, and $\mathbf{K}(\Pi)$ is the spectrum of the spaces $K(\Pi, n)$ we have

COROLLARY. $\tilde{H}_n(X; \mathbf{K}(\Pi)) \approx \tilde{H}_n(X; \Pi)$

3. In order to prove duality theorems, we shall need to introduce cup- and cap-products, and therefore a notion of pairing of two spectra to a third. A *bispectrum* $\mathfrak{E}$ is a double sequence $\{E_{m,n}\}$, together with maps $f_{m,n}\colon E_{m,n} \to \Omega E_{m+1,n}$ and $g_{m,n}\colon E_{m,n} \to \Omega E_{m,n+1}$ such that the diagrams

$$\begin{array}{ccc} E_{m,n} & \xrightarrow{f_{m,n}} & \Omega E_{m+1,n} \\ {\scriptstyle g_{m,n}}\downarrow & & \downarrow{\scriptstyle \Omega g_{m+1,n}} \\ \Omega E_{m,n+1} & \xrightarrow[\Omega f_{m,n+1}]{} & \Omega^2 E_{m+1,n+1} \end{array}$$

are anticommutative. A map of a bispectrum $\mathfrak{E}$ into a spectrum $\mathbf{C}$ is a double sequence of maps $\phi_{m,n}\colon E_{m,n} \to C_{m+n}$ such that the top triangle in the diagram



is commutative, while the bottom is commutative up to a suitable sign.

If **A** and **B** are spectra, then the spaces $A_m \times B_n$ form in a natural way a bispectrum $\mathbf{A} \times \mathbf{B}$; a pairing of **A** with **B** to **C** is a map of the bispectrum $\mathbf{A} \times \mathbf{B}$ into the spectrum **C**. Let **S** be the spectrum of spheres; then there is a natural pairing of **A** with **S** to **A** for any spectrum **A**.

Let X be a simplicial complex, and let A be a subcomplex of X. Let $N(A)$ be the regular neighborhood of A in X, and let $X \div A$ be the closure of the complement of $N(A)$; thus A is a deformation retract of $N(A)$ and $X \div A$ of $X - A$. If further B is a subcomplex of A, let $\pi_1: N(A) \to N(A)/N(B)$, $\pi_2: X \div B \to X \div B/X \div A$ be the natural maps. Then the *reduced diagonal map* $\Delta: X \to N(A)/N(B) \times X \div B/X \div A$ is defined by

$$\Delta(x) = \begin{cases} \pi_1(x) \times \pi_2(x) & (x \in N(A) \cap X \div B) \\ \text{base point} & \text{otherwise} \end{cases}$$

Given a pairing of **A** with **B** to **C**, we can then define cup- and cap-product pairings

$$H^p(A, B; \mathbf{A}) \otimes H^q(X \div B, X \div A; \mathbf{B}) \to H^{p+q}(X; \mathbf{C})$$
$$H^p(A, B; \mathbf{A}) \otimes H_n(X; \mathbf{B}) \to H_{n-p}(X \div B, X \div A; \mathbf{C})$$

and these have many of the formal properties of the usual cup- and cap-products.

4. An orientable n-manifold X is called a Π-*manifold* if and only if its fundamental class is stably spherical; i.e., the natural homomorphism $H_n(X; \mathbf{S}) \to H_n(X; \mathbf{K}(Z))$ is onto. Let X be a triangulated Π-manifold and let $z \in H_n(X; \mathbf{S})$ be an element mapping onto a fundamental class. The cap product with z induces homomorphisms $\phi_p: H^p(A, B; \mathbf{A}) \to H_{n-p}(X \div B, X \div A; \mathbf{A})$ for any subcomplexes $A \supset B$ of X.

THEOREM 2. *The homomorphisms ϕ_p are isomorphism.*

COROLLARY 1. (Alexander duality theorem). *If A is a subcomplex of S^n, then, for any spectrum* **A**,

$$\tilde{H}^p(A; \mathbf{A}) \approx \tilde{H}_{n-p-1}(S^n - A; \mathbf{A})$$

COROLLARY 2. (Poincaré duality theorem for Π-manifolds). *If X is a triangulable Π-manifold of dimension n, then $H^p(X; \mathbf{A}) \approx H_{n-p}(X; \mathbf{A})$ for any spectrum* **A**.

Remark: The isomorphism of Corollary 2 is natural for maps of spectra (once the class z has been chosen). Conversely it is easy to see that, if the orientable n-manifold satisfies Poincaré duality functorially, then X is a Π-manifold.

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[1] The notion of spectrum was introduced by Lima (University of Chicago dissertation, 1958); cf. Spanier, E. H., *Ann. Math.*, **70**, 338–378 (1959).

CONTRIBUTIONS TO INFORMATION THEORY*

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The purpose of this note is to describe a number of new results in coding theory. The proofs will be given elsewhere. The results fall into two not directly related groups, consisting, respectively, of the first three sections and the last three sections. Wherever feasible the terminology of specific references will be adopted so as to avoid the need of often lengthy definitions.

1. *Channels with Side Information at the Receiver.*—We consider the channel described by Shannon,[1] but in the situation where (what he calls) the state of the channel at each letter is *known to the receiver but not to the sender.* (The reverse situation is treated by Shannon.) The definitions and notation of Shannon[1] will be adopted *in toto.* The present problem and the problems treated by Shannon are related to the problems treated by the author in a paper to appear shortly.[2]

By a probability a-vector $\pi = (\pi_1, \ldots, \pi_a)$ we mean a vector with a nonnegative components which sum to one. Let G be the set of pairs (t, j), $t = 1, \ldots, h$; $j = 1, \ldots, b$. Let $q_1(\cdot, \cdot \mid \pi)$ be a distribution on G which depends on π as a parameter and is defined as follows for any pair (t, j) and any π:

$$q_1(t, j|\pi) = \sum_{i=1}^{a} \pi_i g_i p_{ti}(j)$$

Let $q_2(\cdot, \cdot \mid i)$ be a distribution on G which depends on i, $i = 1, \ldots, a$, as a parameter, and is defined as follows for any pair (t, j) and any i:

$$q_2(t, j \mid i) = g_i p_{ti}(j)$$

A code (n, N, λ) is a block code with N messages, block length equal to n, and probability $\leq \lambda$ that any message transmitted will be incorrectly decoded.

Let $H(q)$ denote the entropy of the distribution q. It is shown that the capacity C_1 of the present channel is given by

$$C_1 = \max_{\pi} [H(q_1(\cdot, \cdot|\pi) - \sum_{i=1}^{a} \pi_i H(q_2(\cdot, \cdot|i)] \tag{1}$$

The following coding theorem and strong converse hold for this channel:

THEOREM 1. *Let λ, $0 < \lambda < 1$, be arbitrary. There exists a number $K_1 > 0$ such that, for any n, there exists a code $(n, 2^{nC_1 - K_1\sqrt{n}}, \lambda)$, and there does not exist a code $(n, 2^{nC_1 + K_1\sqrt{n}}, \lambda)$.*

2. *Coding Theorems for Simultaneous Channels.*—For any n let $P_n^{(1)}, \ldots, P_n^{(t)}$ be channel probability functions (c.p.f.'s). Any of these may govern the transmission of a word (block) of n letters, and the choice of c.p.f. varies *arbitrarily* from word to word.

We consider first the case where neither sender nor receiver knows the c.p.f. being used to govern the transmission of any word. Let Q_n' be any stochastic input on sequences of n letters, $Q_n''^{(i)}$ be the stochastic output for the ith c.p.f.,

and let $Q_n^{(i)}$ denote the joint distribution of input and output sequences (for the ith c.p.f.). Define

$$I_n^{(i)}(u, v) = \log_2 \left(\frac{Q_n^{(i)}(u, v)}{Q_n'(u) Q_n'^{(i)}(v)} \right).$$

For the simultaneous channel described above the following theorem holds:

THEOREM 2. *Let θ and $\lambda < 1$ be any positive numbers. There exists an (n, N, λ) code for the simultaneous channel such that*

$$N > 2^{\theta} \left[\frac{\lambda}{t} - \sum_{i=1}^{t} Q_n^{(i)} \{(u, v) \mid I_n^{(i)}(u, v) \leq \theta\} \right]$$

Now consider the channel of Theorem 2 modified so that the c.p.f. which governs the transmission of any word is *known to the sender but not to the receiver.* Let $Q_n'^{(1)}, \ldots, Q_n'^{(t)}$ be any t stochastic inputs on sequences of n letters. Define

$$\bar{I}_n^{(i)}(u, v) = \log_2 \left(\frac{Q_n^{(i)}(u, v)}{Q_n'^{(i)}(u)\, Q_n''^{(i)}(v)} \right)$$

For the simultaneous channel described above the following theorem holds:

THEOREM 3. *Let θ and $\lambda < 1$ be any positive numbers. There exists an (n, N, λ) code for the simultaneous channel such that*

$$N > 2^{\theta} \left[\lambda - \sum_{i=1}^{t} Q_n^{(i)} \{(u, v) \mid \bar{I}_n^{(i)}(u, v) \leq \theta\} \right].$$

The special case of Theorem 2 for $t = 1$ is due to Shannon.[3] A result similar to Theorem 2 was proved by Blackwell, Breiman, and Thomasian.[4] Theorem 3 is connected with certain results of the author.[2]

3. *Weak Converses of Coding Theorems.*—For any n let $\{P_n^{(s)}, s \in S\}$ be a (not necessarily finite) collection of c.p.f.'s. Let Q_n' be, as in Section 2, a stochastic input on sequences of n letters. For $P_n^{(s)}$ let $R_n^{(s)}(Q_n')$ denote Shannon's rate of transmission (the expected value of $I_r^{(s)}$ of Section 2). Define

$$C'(n) = \max_{Q_n'} \inf_{s} R_n^{(s)}(Q_n'),$$

and

$$C''(n) = \inf_{s} \max_{Q_n'} R_n^{(s)}(Q_n').$$

The following theorems hold:

THEOREM 4. *For any simultaneous channel (which may have infinitely many c.p.f.'s) where (1) neither sender nor receiver knows the (arbitrarily chosen) c.p.f. which governs the transmission of any word, or (2) the receiver but not the sender knows the (arbitrarily chosen) c.p.f. which governs the transmission of any word, any code (n, N, λ) satisfies*

$$\log N < \frac{C'(n) + 1}{1 - \lambda}.$$

(Theorem 4 includes the so-called weak converse of Fano, cited in Feinstein,[5] p. 44.)

THEOREM 5. *For any simultaneous channel (which may have infinitely many c.p.f.'s) where the sender but not the receiver knows the (arbitrarily chosen) c.p.f. which governs the transmission of any word, any code* (n, N, λ) *satisfies*

$$\log N < \frac{C''(n) + 1}{1 - \lambda}.$$

4. *The Discrete Finite Memory Channel.*—The discrete finite memory channel (d.f.m.c.) is defined in Feinstein,[5] (p. 90). Let m be the memory of the channel, and suppose, only for simplicity of exposition, that both input and output alphabets have a symbols, say $1, \ldots, a$. (We shall make this assumption, also only for simplicity of exposition, in Sections 5 and 6.)

Let l be any positive integer. Consider the discrete *memoryless* channel (d.m.c.) whose input alphabet consists of the blocks of length $(l + m)$ in the symbols $1, \ldots, a$, whose output alphabet consists of the blocks of length l in the symbols $1, \ldots, a$, and whose c.p.f. is defined as follows: The probability that, when the symbol $b = (b_1, \ldots, b_{(l+m)})$ is sent, the symbol $c = (c_1, \ldots, c_l)$ will be received, is equal to the probability that, in the original d.f.m.c., when the block b is sent, the last l symbols of the received block of length $(l + m)$ will be c. Let $C(l + m)$ be the capacity of this d.m.c. Then the following theorem holds:

THEOREM 6. *We have*

$$\lim_{l \to \infty} \frac{C(l + m)}{(l + m)} = \sup_l \frac{C(l + m)}{(l + m)} = C_3 \text{ (say)}, \tag{2}$$

and, for any l,

$$\frac{C(l + m)}{(l + m)} \leq C_3 \leq \frac{C(l + m)}{(l + m)} + \frac{m \log_2 a}{(l + m)}. \tag{3}$$

The number C_3 is the capacity of the d.f.m.c. Because of equation (3) it is in principle computable to within any specified positive error. For the d.f.m.c. the following coding theorem and strong converse hold:

THEOREM 7. *Let* λ, $0 < \lambda < 1$, *and* $\epsilon > 0$ *be arbitrary. For* n *sufficiently large there exists a code* $(n, 2^{n(C_3 - \epsilon)}, \lambda)$, *and there does not exist a code* $(n, 2^{n(C_3 + \epsilon)}, \lambda)$.

Theorems 6 and 7 generalize results of the author[6] and of Feinstein.[7] The d.f.m.c. could be treated as a simultaneous channel by Theorems 2 and 4, and a coding theorem and weak converse obtained. These results would not be as strong as Theorems 6 and 7.

5. *A Channel with Infinite Memory on the Previously Transmitted Letters.*—Let $w(j|i) \geq \alpha > 0$ for $i, j = 1, \ldots, a$, and let

$$\sum_{j=1}^{a} w(j|i) = 1, \; i = 1, \ldots, a.$$

Let $\gamma_1, \gamma_2, \ldots$ be an infinite sequence of nonnegative numbers such that

$$\sum_{i=1}^{\infty} \gamma_i = 1, \; \sum_{i=1}^{\infty} i \, \gamma_i < \infty.$$

Write

$$r_i = \sum_{j=i}^{\infty} g_j.$$

Let $s = (\ldots, s_{-2}, s_{-1}, s_0)$ be any infinite sequence of integers, each one of $1, \ldots, a$, which has been sent over the stationary, nonanticipating channel. If now the integer j_1 is sent over the channel, the probability that the integer j_2 will be received does not depend on the previously received integers and is given by

$$w(j_2|j_1)g_1 + \sum_{i=0}^{\infty} w(j_2|s_{-i})g_{i+2}. \tag{4}$$

Let s^* be any fixed sequence s. Let m and l be any positive integers. Let $C(l + m)$ be the capacity of the d.m.c. channel described in Section 4, with the probabilities involved calculated according to equation (4) with $s = s^*$. (Note the different definition of m.)

Define

$$\Phi(m, l) = \prod_{i=m+1}^{m+l} \frac{\alpha + r_{(i+1)}}{\alpha}$$

$$h_1(m, l) = \max \{(\Phi(m, l) \log \Phi(m, l) - 1), (1 - [\Phi(m, l)]^{-1} \log \Phi(m, l))\}$$

$$h_2(m, l) = \Phi(m, l) - 1$$

$$\Delta(m \cdot l) = 2h_2(m, l) \log_2 a + \frac{2h_1(m, l) + m \log_2 a}{(m + l)}$$

The following theorem holds for the channel of this section:

THEOREM 8. *We have that*

$$\lim \frac{C(m + l)}{(m + l)} = C_4 \text{ (say)} \tag{5}$$

as $m \to \infty$, $m/l \to 0$. *For any* m *and* l *we have*

$$\left| C_4 - \frac{C(m + l)}{(m + l)} \right| < \Delta(m, l). \tag{6}$$

The number C_4 is the capacity of the channel of this section. Because of equation (6), C_4 is, in principle, computable to within any specified positive error. For the channel of this section the following coding theorem and strong converse hold:

THEOREM 9. *Let* λ, $0 < \lambda < 1$, *and* $\epsilon > 0$ *be arbitrary. For* n *sufficiently large there exists a code* $(n, 2^{n(C_4 - \epsilon)}, \lambda)$, *and there does not exist a code* $(n, 2^{n(C_4 + \epsilon)}, \lambda)$.

6. *A Channel with Infinite Memory on the Previously Received Letters.*—Suppose given a stochastic $a \times a$ matrices $Z(1), \ldots, Z(a)$, with $z(i, j, t) \geq \beta > 0$ the element in the jth row and tth column of $Z(i)$. Let the letter j_1 be sent over the channel when s_0 was the last *received* letter. The probability that j_2 will be received does not depend on the letters previously transmitted or previously received except s_0, and is given by

$$z(j_1, s_0, j_2). \tag{7}$$

Define

$$\theta = \max_i [1 - \sum_t \min_j z(i, j, t)].$$

Then the capacity C_5 of the channel of the present section is given by the same process as C_4, except that now $\Phi(m, l) = 1 + (\theta^m/\beta)$, and, in the definition of $C(m + l)$,

the place of s^* is taken by an arbitrarily fixed number s' which is put for s_0 in equation (7). For the channel of the present section Theorems 8 and 9 then hold verbatim with C_4 replaced by C_5.

For a channel slightly more general than that of this section a noncomputable definition of C_5 was given by Blackwell, Breiman, and Thomasian,[8] who proved a coding theorem and weak converse. (Brief proofs of these are now available, for the coding theorem is a special case of Theorem 2, and the weak converse is a special case of Theorem 4.) The new results of this section are the definition of C_5, the result corresponding to equation (6), and the strong converse.

By the same methods by which the present results follow, one could treat the channel where equation (7) is replaced by an expression analogous to (4) in all the previously received letters. Moreover, one can combine the results of the present and immediately preceding sections to obtain the corresponding results when the probability which corresponds to equations (4) and (7) involves all the previously received and transmitted letters in the analogous manner. The formal proofs of these are entirely routine.

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SOME STATISTICAL OBSERVATIONS ON A COOPERATIVE STUDY OF HUMAN PULMONARY PATHOLOGY. III

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The difference between precision and accuracy could hardly be better stated than it was by Simon Newcomb in summing up his determination of the velocity of light.[1] He had given his value of the velocity of light *in vacuo* as 299,860 km per second and Michelson's subsequent value as 299,853, and then stated: "So far as could be determined from the discordance of the separate measures, the mean error of Newcomb's result would be less than 10 km. But making allowance for the various sources of systematic error the actual probable error was estimated at ±30 km."

Precision is a mathematical concept attaching to a series of observations; accuracy is a scientific concept taking into account the probable reproducibility of the result by other equally competent scientists. Precision estimates guard against one's sample being too small, but not at all against its being biased. If the sample is small enough, the "sampling error" may be large enough to cover up any likely bias; if the sample is large, it is very likely to be biased enough, in one way or another, to make the accuracy less than the precision. This Newcomb believed was the case with his determination of the velocity of light, and he was a good enough scientist to desire to claim no more than he believed to be true.[2]

TABLE 1

Line	A	B	C	D	E	F	G	H	I	J	K	L	S	S′	V
1	1	0	0	0	0	1	0	0	0	0	1	0	3	3	27
2	1	0	0	3	0	1	0	0	0	0	1	0	6	12	108
3	0	1	0	1	1	3	0	0	0	0	1	0	7	13	107
4	1	2	1	1	1	1	1	1	0	0	1	0	10	12	44
5		1	1	1	2	1	1	1	1	1	1	0	12	14	24
6	1	2	1	1	1	1	1	1	1	1	1	1	13	15	11
7	2	2	2	1	2	1	1	0	1	1	1	0	14	22	68
8	2	2	2	1	2	2	2	1	1	0	0	0	15	27	99
9	2	2	1	3	1	1	1	1	1	1	1	0	15	25	75
10	1	2	1	3	3	1	1	1	1	0	1	1	16	30	104
11	1	2	1	3	0	1	2	0	2	2	1	2	17	33	107
12	2	2	3	1	1	1	2	0	0	3	1	1	17	35	131
13	2	2	1	3	3	1	1	1	1	1	1	0	17	33	107
14	1	2	2	2	2	2	2	0	2	0	2	2	19	37	83
15	2	2	1	3	2	2	2	2	1	1	1	0	19	37	83
16	2	2	2	2	2	2	2	1	1	2	2	0	20	38	56
17	2	2	1	3	2	1	2	2	1	1	1	2	20	38	56
18	0	2	2	2	2	1	2	2	2	2	2	2	21	41	51
19	2	2	2	3	2	1	2	1	0	2	2	2	21	43	75
20	2	2	2	2	2	2	2	2	2	0	2	2	22	44	44
21	1	1	2	3	2	2	2	2	2	2	1	2	22	44	44
22	2	2	2	2	2	1	2	2	1	2	2	2	22	42	20
23	2	3	2	3	3	1	2	1	1	1	2	2	23	51	83
24	2	2	3	1	1	2	2	2	2	2	2	2	23	47	35
25	2	2	1	3	3	2	2	2	1	2	1	2	23	49	59
26	2	2	2	2	2	2	2	1	2	2	2	2	23	45	11
27	2	2	2	2	2	2	2	2	2	2	1	2	23	45	11
28	2	2	2	2	2	2	2	2	2	2	2	2	24	48	0
29	2	2	2	2	2	2	2	2	2	2	2	2	24	48	0
30	2	3	3	3	3	1	3	1	1	3	0	2	25	65	155
31	2	2	2	3	2	2	2	2	2	2	2	2	25	53	11
32	2	2	2	3	3	2	2	3	2	1	2	2	26	60	44
33	2	2	2	2	3	2	2	2	2	2	3	2	26	58	20
34	2	2	2	3	2	2	2	3	2	2	2	2	26	58	20
35	2	2	3	3	3	2	2	2	2	3	1	2	27	65	51
36	1	3	3	3	4	4	1	2	1	2	2	1	27	75	171
37	2	2	3	3	3	3	3	3	3	3	3	3	34	98	20
38	4	4	3	4	4	3	4	4	4	3	3	4	44	164	32
39	4	4	4	3	4	4	4	4	4	4	4	4	47	185	11
40	4	4	4	3	4	4	4	4	4	4	4	4	47	185	11
S	72	82	75	92	85	72	74	63	60	64	65	61	865	2037	2269
S′	158	194	177	244	223	160	170	147	132	150	137	145	2037		
V	1136	1036	1455	1296	1695	1216	1324	1911	1680	1904	1255	2079	17987		

The data have been given for the 12 readings on the 40 slides with the computation form used for checking, viz.: The column (or row) marked S contains the sum of the ratings in the rows (or columns), that marked S′ contains the sum of the squares of the ratings. The over-all sum checks at 865, and the sum of squares at 2037. The column (or row) marked V is computed as $12\,S' - S^2$ (or $40\,S' - S^2$) so as to remain in whole numbers and is therefore 144 times the variance in the row (or, respectively, 1,600 times the variance in the column).

The distinction between precision and accuracy, between accidental and systematic errors is old enough in the so-called exact sciences to have found a place long ago in the leading dictionaries.[3] It is, however, not emphasized as much as it should be in the textbooks on statistics. Although all sciences advance by introducing better definitions and better methods, and thus reduce guesswork, estimate, and personal opinion to narrower limits, none has succeeded in eliminating them, or is likely to, and least of all those whose subjects require the use of statistics in complicated situations in which measurement and even classification is far from precise, where causation is not known but sought, and error too often has social implications of right or wrong instead of merely its scientific significance.

TABLE 2

Line	A	B	C	D	E	F	G	H	I	J	K	L	V
1	9	−6	−3.9	−9	−6.9	9	−3.6	−0.3	0.6	−0.6	11.1	0.3	39.9
2	6	−9	−6.9	24	−9.9	6	−6.6	−3.3	−2.4	−3.6	8.1	−2.7	85.1
3	−7	2	−7.9	−1	1.1	29	−7.6	−4.3	−3.4	−4.6	7.1	−3.7	94.3
4	2	11	1.1	−4	−1.9	2	1.4	4.7	−6.4	−7.6	4.1	−6.7	27.9
5	0	−3	−0.9	−6	8.1	0	−0.6	2.7	3.6	2.4	2.1	−8.7	18.2
6	−1	8	−1.9	−7	−4.9	−1	−1.6	1.7	2.6	1.4	1.1	2.3	13.6
7	10	7	9.1	−8	6.1	−2	−2.6	−11.3	1.6	0.4	0.1	−10.7	49.1
8	9	6	8.1	−9	5.1	9	8.4	−0.3	0.6	−12.6	−12.9	−11.7	75.3
9	9	6	−3.9	15	−6.9	−3	−3.6	−0.3	0.6	−0.6	−0.9	−11.7	47.1
10	−4	5	−4.9	14	16.1	−4	−4.6	−1.3	−0.4	−13.6	−1.9	−0.7	62.4
11	−5	4	−5.9	13	−20.9	−5	6.4	−14.3	10.6	9.4	−2.9	10.3	105.6
12	7	4	18.1	−11	−8.9	−5	6.4	−14.3	−13.4	21.4	−2.9	−1.7	126.0
13	7	4	−5.9	13	15.1	−5	−5.6	−2.3	−1.4	−2.6	−2.9	−13.7	63.6
14	−7	2	4.1	−1	1.1	5	4.4	−16.3	8.6	−16.6	7.1	8.3	70.9
15	5	2	−7.9	11	1.1	5	4.4	7.7	−3.4	−4.6	−4.9	−15.7	51.7
16	4	1	3.1	−2	0.1	4	3.4	−5.3	−4.4	6.4	6.1	−16.7	38.6
17	4	1	−8.9	10	0.1	−8	3.4	6.7	−4.4	−5.6	−5.9	7.3	38.0
18	−21	0	2.1	−3	−0.9	−9	2.4	5.7	6.6	5.4	5.1	6.3	59.4
19	3	0	2.1	9	−0.9	−9	2.4	−6.3	−17.4	5.4	5.1	6.3	51.6
20	2	−1	1.1	−4	−1.9	2	1.4	4.7	5.6	−19.6	4.1	5.3	42.9
21	−10	−13	1.1	8	−1.9	2	1.4	4.7	5.6	4.4	−7.9	5.3	42.3
22	2	−1	1.1	−4	−1.9	−10	1.4	4.7	−6.4	4.4	4.1	5.3	21.3
23	1	10	0.1	7	9.1	−11	0.4	−8.3	−7.4	−8.6	3.1	4.3	48.3
24	1	−2	12.1	−17	−14.9	1	0.4	3.7	4.6	3.4	3.1	4.3	61.5
25	1	−2	−11.9	7	9.1	1	0.4	3.7	−7.4	3.4	−8.9	4.3	38.1
26	1	−2	0.1	−5	−2.9	1	0.4	−8.3	4.6	3.4	3.1	4.3	14.1
27	1	−2	0.1	−5	−2.9	1	0.4	3.7	4.6	3.4	−8.9	4.3	15.3
28	0	−3	−0.9	−6	−3.9	0	−0.6	2.7	3.6	2.4	2.1	3.3	8.6
29	0	−3	−0.9	−6	−3.9	0	−0.6	2.7	3.6	2.4	2.1	3.3	8.6
30	−1	8	10.1	5	7.1	−13	10.4	−10.3	−9.4	13.4	−22.9	2.3	118.6
31	−1	−4	−1.9	5	−4.9	−1	−1.6	1.7	2.6	1.4	1.1	2.3	7.6
32	−2	−5	−2.9	4	6.1	−2	−2.6	12.7	1.6	−11.6	0.1	1.3	33.5
33	−2	−5	−2.9	−8	6.1	−2	−2.6	0.7	1.6	0.4	12.1	1.3	25.1
34	−2	−5	−2.9	4	−5.9	−2	−2.6	12.7	1.6	0.4	0.1	1.3	22.1
35	−3	−6	8.1	3	5.1	−3	−3.6	−0.3	0.6	11.4	−12.9	0.3	38.7
36	−15	6	8.1	3	17.1	21	−15.6	−0.3	−11.4	−0.6	−0.9	−11.7	131.7
37	−10	−13	1.1	−4	−1.9	2	1.4	4.7	5.6	4.4	4.1	5.3	34.5
38	4	1	−8.9	−2	0.1	−8	3.4	6.7	7.6	−5.6	−5.9	7.3	33.2
39	1	−2	0.1	−17	−2.9	1	0.4	3.7	4.6	3.4	3.1	4.3	31.5
40	1	−2	0.1	−17	−2.9	1	0.4	3.7	4.6	3.4	3.1	4.3	31.5
V	40.2	30.7	38.2	86.7	58.2	59.4	20.6	46.6	37.8	62.5	46.5	50.7	48.2

In the table are given the 480 residuals E_{ps}, each multiplied by 12 and the product diminished by 1/40 = 0.025 to keep the calculations simpler. The rows therefore have the sum −0.3 and the columns −1.0 instead of zeros. The column and the row marked V give the respective variances in the rows and columns reduced to one decimal place, the mean of each being 48.2, which gives 0.3347 on division by 144 and thus agrees as well as can be expected with the stated value 0.3345.

What made the exact sciences exact was the careful attention to definition, classification, measurement, and the reduction of observations, and it is this that is making the inexact sciences less inexact. Our first paper discussed and illustrated the extent to which the readings of the pathologists still differed after some conferences devoted to discussing definitions. It was clear that the remaining differences were sufficient to make comparability of their respective findings unsafe unless information was obtained which would "standardize" the results. In our second paper the means and method of standardization were set forth.[4] In the present note we return to the problem of the further examination of the readings of the 40 slides using a simple type of variance analysis.[5]

Table 1 gives the data.[6] Clearly the ratings depend in part on the slides read as is seen by the variation in the columns and in part on the pathologist doing the reading as is shown by the variation in the rows. If F_{ps} represents the rating of the slide s, with $s = 1, 2, \ldots, 40$, by the pathologist p, with $p = 1, 2, \ldots, 12$, it is obvious that F_{ps} depends in part on p and in part on s. If we average the ratings of the 12 pathologists on slide s we get a mean m_s, and if we average the ratings of each pathologist on the 40 slides we get the mean m_p. It is a natural, and indeed the usual, assumption to consider the mean as the best value we can get for a variable and to consider deviations from the mean as errors in the statistical sense (not as mistakes). Thus $F_{ps} - m_s$, $p = 1, 2, \ldots, 12$, will be the 12 errors of the 12 pathologists on slide s, and $F_{ps} - m_p$, $s = 1, 2, \ldots, 40$, will be the 40 errors (deviations) of the 40 slides according to pathologist p.

The mean M of all the $ps = 480$ ratings F_{ps}, the mean of the 40 values m_s, and the mean of the 12 values m_p all have to be equal. We can "correct" each rating F_{ps} to the means m_s and m_p by writing

$$E_{ps} = F_{ps} - M - (m_s - M) - (m_p - M) = F_{ps} - m_s - m_p + M.$$

That is to say, E_{ps} represents the accidental or residual variation in the rating after taking out the systematic parts due to the rating habits of the pathologists and to the pathology of the slides. It is a simple theorem in elementary algebra that the total variance of F (i.e., the standard deviation squared of $F_{ps} - M$) is equal to the variance of m_s plus the variance of m_p plus the variance of the residuals E_{ps}.

For the data of Table 1 these variances are

$$\sigma^2 F_{ps} = 0.9962, \qquad \sigma^2 m_s = 0.6023, \qquad \sigma^2 m_p = 0.0594, \qquad \sigma^2 E_{ps} = 0.3345.$$

The sum of the last three is the first, and if we are willing to use variances to estimate the relative contributions of each of the last three to the total which is the first, we find that 60.5 per cent, 6.0 per cent, and 33.5 per cent, respectively, of the total variance is due to the differences in the slides, the differences in the pathologists, and to all other sources.[7] The corresponding standard deviations are

$$\sigma F_{ps} = 0.9981, \qquad \sigma m_s = 0.7760, \qquad \sigma m_p = 0.2438, \qquad \sigma E_{ps} = 0.5783.$$

However, the standard deviations have not the additive property that the variances have and there is no accepted way of distributing the first among the last three.

To obtain the variance of the 480 residuals E_{ps} it is not necessary to find them

individually; it is sufficient to find the variances of the original distribution, of the means of the rows, and of the means of the columns, and use the summation theorem. However, there is some interest in looking at the residuals and they are given in Table 2. The variances in the rows may be taken as measures of disagreement and hence of the difficulty of reading the slides. Although the average of these variances in Table 2 at 48.2 is less by about one seventh than the 56.7 in Table 1, there are about one third of the slides for which the variance in Table 1 is somewhat less than in Table 2. This means that if one tries to indicate the serial order of difficulty of reading the slides by the order of the variances, he will have first to decide whether he will use the crude variances or those adjusted to the mean ratings of the different readers.

The original ratings were distributed essentially normally in five groups; the adjusted residuals are in nearly 160 groups making the distribution more continuous. The standard deviation of the scaled-up values in Table 2 is 6.94. Expressed in terms of the standard deviation as a unit the distribution of the E's is compared in Table 3 with that of a normal one. It is seen that the E's are not normally dis-

TABLE 3

Scale	<-3	-3 to -2	-2 to -1	-1 to -0.5	-0.5 to -0.2	-0.2 to 0
E's	3	14	53	57	55	34
Normal	0.6	10.3	65	72	54	38
Scale	0 to 0.2	0.2 to 0.5	0.5 to 1	1 to 2	2 to 3	>3
E's	63	61	79	51	6	4
Normal	38	54	72	65	10.3	0.6

tributed. There are too many deviations greater than 3σ, whether positive or negative, too few in the middle ranges on both sides, and too many small positive ones. This departure from normality is not unusual, but it does make estimates of probability somewhat doubtful; we shall proceed descriptively.

First we modify the variances calculated above for the means of the 40 slides, the means of the 12 pathologists, and the 480 residuals by multiplying them respectively by the numbers in the samples and then dividing by the degrees of freedom which are 39, 11, and 429, respectively. We thus obtain the estimated variances of the universes to which the three samples belong, namely, 0.6178, 0.0648, and 0.3743; the corresponding square roots are 0.7860, 0.255, 0.6118. If we use the last as the best estimate of the standard deviation of the residual "random" variations, a random sample of 40 will have its mean subject to a standard deviation of 0.0967, whereas the estimated standard deviation of the means of the pathologists at 0.255 is 2.63 times as much, which shows that the pathologists differ much more than they would by chance. Similarly, the mean of 12 random elements would have a standard deviation of 0.1766, so that the 40 slides differ 4.45 times as much as they would by chance.[8]

[1] *Encyclopedia Britannica*, 11th ed., vol. 16, p. 625.

[2] Actually Newcomb was not conservative enough. Michelson's 1926 value was 299,796 ± 1. (See, *Encyclopedia Britannica*, 14th ed., vol. 23, p. 37.) Michelson let Newcomb's statement stand and made no estimate of his own accuracy; the ±1 was his precision. Many of Michelson's collaborators and others in the next 15 years made determinations which ran around 20 km/sec less than his, and R. T. Birge, an undoubted authority in precision analysis, reviewing all the evidence, in 1941 set the value at 299,776 ± 4. More recently DuMond and Cohen have set the

figure 299,792.9 ± 0.8, which is back close to Michelson's of 1926, but statistically inconsistent with Birge's. The long history of the determinations of this important constant gives little ground for belief that precision estimates are safe guides to scientific accuracy.

[3] See, for example, *Webster's New International Dictionary* under "Error, 9. Math."

[4] These PROCEEDINGS, **43**, 1073–1078 (1957), and **45**, 389–393 (1959).

[5] The type which we shall use is only the simplest, the one which is the first step in the analysis of sets of grades assigned by different readers to the answers of a group of students upon examinations, especially when the questions call for the essay type of answer. We do not have here to examine variation in a complicated system where one computes the trend of a presumed effect on a variable, or some of the variables, which represent presumed causes; but it is necessary in that case also to go on to some sort of variance analysis before one can assign even provisionally the putative amounts of causation to the individual variables or the pairs jointly. An illustration of this can be found in E. L. Thorndike's *Your City* (Harcourt and Brace, 1939). In such cases it is important to find how much of the total variance of the "effect" is accounted for by the "causal" variables used and how much remains unaccounted for and to verify that the latter is but a small fraction of the whole.

[6] We follow the classification of our second paper in five groups: 0, normal; 1, hyperplasia; 2, metaplasia; 3, atypical metaplasia and carcinoma-in-situ; 4, carcinoma. The numbers in these five classes are respectively: 48, 120, 223, 57, 32; the mean is 1.80208, the variance is 0.99625, the skewness is 0.21 ± 0.11, and the kurtosis is −0.06 ± 0.22.

[7] Among the sources should be counted the differing standard deviations of the different pathologists, for sets of ratings may differ considerably in their scatter about the mean. We understand that Professor C. F. Mosteller of Harvard has a method of allowing for this scatter, but we shall not go into this.

[8] There is no simple way to assign probabilities to the figures 2.63 and 4.45 of the ratios of the actual observed standard deviations to the hypothetical chance ones; but these ratios are in fact the square roots of the ratios F of mean squares for which the $P = .05$ and $P = .01$ values have been tabulated in G. W. Snedecor's *Statistical Methods Applied to Experiments in Agriculture and Biology* (chap. 10, 4th ed., 1946). The $P = .01$ values of F are respectively 2.29 and 1.63 which are much smaller than the squares of 2.63 and 4.45. Had we decided to carry through a standard type of variance analysis, we should have avoided the longer calculation of the standard deviations and their ratios; but we considered that standard deviations are more familiar to medical scientists than variance analysis as usually presented.

ON A THEORY OF DISPERSION BY CONTINUOUS MOVEMENTS

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1. *Introduction.*—One of the central relations in the theory of turbulent diffusion is Richardson's law of dispersion.[1] It has often been derived from Kolmogoroff's theory[2] with the help of dimensional reasoning.[3, 4] But reservations on the range of applicability of this derivation have also been expressed.[3, 5] In the present note, we show how Richardson's law can be derived by applying the general approach of Taylor's theory of diffusion by continuous movements[6] when we consider dispersion from a point source. This derivation may be valid even when not all the requirements of Kolmogoroff's theory are fulfilled.

The analysis gives rise to a Lagrangian parameter B which is of the same dimension as the rate of energy dissipating ϵ. However, unlike previous analyses involving ϵ, the parameter B is now defined by a different physical process, and it is

not necessarily identifiable with ϵ (numerical factors being allowed). The parameter B also enters the dispersion relations with definite numerical coefficients. In this respect, the gain in our theoretical understanding is similar to that attained by Taylor's precise definition of the turbulent diffusion coefficient.

The results obtained in this paper are comparable with those of Obukhov,[7] who, however, started with the assumption that "the evolution of the state of the selected particle in time forms a Markov process and can be described by the Fokker-Planck equation in the space (x, v)." Such a restrictive assumption is not made in this paper. But it is clear that the two theories are related, and that our parameter B is essentially the diffusion coefficient in the velocity space in the Fokker-Planck equation, if the latter is indeed applicable. The exact relationship between the present results and Obukhov's theory will be discussed in a more detailed communication by referring to the general theory of Brownian motion (see Uhlenbeck and Ornstein, and Chandrasekhar[8]).

Obukhov also assumed that his parameter B is proportional to ϵ with a constant coefficient of the order of unity. Such an assumption is certainly plausible from the point of view of Kolmogoroff's theory. However, it is also possible that the ratio B/ϵ is a function of the Reynolds number of turbulence R_λ and some other dimensionless parameter. An attempt to find this ratio might be a crucial step in connecting the Eulerian and the Lagrangian descriptions of turbulence. It appears that experiments can be set up for various values of R_λ to test (with B determined from equation (15)) whether there is a universal relationship of the form $B/\epsilon = f(R_\lambda)$.

The theory has been extended to the case of homogeneous anisotropic turbulence so that the horizontal diffusion coefficient may even be many times different from the vertical. This brings out more clearly the difference between the present approach and Kolmogoroff's theory, in which the concept of local isotropy plays an important role. The details of this anisotropic theory will be presented in later communications.

2. *One-Particle Analysis.*—Consider first the motion of a single particle released in a stationary homogeneous field of turbulent motion. Instead of considering the statistical distribution of the particles in space for an ensemble of such experiments, as Taylor did in his original theory of diffusion by continuous movements, we apply that approach to the determination of the velocity correlation of the same particle at an interval of time τ apart. Indeed, we know that the velocity $\mathbf{u}(t)$ may be obtained by an integration of the acceleration $\mathbf{a}(t)$ so that

$$\mathbf{u}(t + \tau) - \mathbf{u}(t) = \int_t^{t+\tau} \mathbf{a}(t')dt'$$

Thus,

$$\frac{1}{2}\frac{d}{d\tau}\overline{|\mathbf{u}(t+\tau) - \mathbf{u}(t)|^2} = \int_t^{t+\tau} \overline{\mathbf{a}(t+\tau)\cdot\mathbf{a}(t')}\, dt'$$

where the overhead bar denotes the ensemble average. If we now introduce the correlation coefficient $R_0(\tau)$ for the acceleration

$$\overline{\mathbf{a}(t+\tau)\cdot\mathbf{a}(t)} = \overline{|\mathbf{a}|^2}R_0(\tau), \qquad (1)$$

we obtain

$$\frac{1}{2}\frac{d}{d\tau}\overline{|\mathbf{u}(t+\tau)-\mathbf{u}(t)|^2}=\overline{|\mathbf{a}|^2}\int_0^\tau R_0(s)\,ds. \tag{2}$$

Suppose that $R_0(\tau)$ is small for $\tau \geq \tau_1$, then for $\tau > \tau_1$, we may approximate the above relation by

$$\frac{1}{2}\frac{d}{d\tau}\overline{|\mathbf{u}(t+\tau)-\mathbf{u}(t)|^2}=B_0,\ B_0=\overline{|\mathbf{a}|^2}\int_0^{\tau_1} R_0(s)\,ds. \tag{3}$$

Thus,

$$\overline{|\mathbf{u}(t+\tau)-\mathbf{u}(t)|^2}=2B_0\tau,\ \text{for}\ \tau \gg \tau_1. \tag{4}$$

Clearly this implies that τ, while large compared with τ_1, must be appropriately small in a certain sense. This is exactly the kind of requirement in Kolmogoroff's theory.[9] Indeed, equation (4) has often been written down by dimensional arguments based on Kolmogoroff's theory, with B replaced by ϵ, and with an indefinite numerical coefficient. However, it appears that not all the requirements in Kolmogoroff's theory, such as an extremely large Reynolds number of turbulence, must be fulfilled to make the present derivation valid.

3. *Two-Particle Analysis.*—Consider now two particles simultaneously released in a turbulent wind. They will meander together with the wind according to the large-scale motions, but will also drift apart due to the small-scale motions. It is the latter we are interested in.

Let the initial coordinates of the particles be $\mathbf{x}_1^{(0)}$ and $\mathbf{x}_2^{(0)}$. After a time interval t, let their coordinates be $\mathbf{x}_1$ and $\mathbf{x}_2$ and their velocities $\mathbf{u}_1$ and $\mathbf{u}_2$. Then their separation $\mathbf{x}=\mathbf{x}_2-\mathbf{x}_1$ and their relative velocity $\mathbf{v}=\mathbf{u}_2-\mathbf{u}_1$ bear the relationship

$$\mathbf{v}=\frac{d\mathbf{x}}{dt}. \tag{5}$$

If, now, the particles are released at such small distances apart that their initial separation $\mathbf{x}^0$ and their initial relative velocity $\mathbf{v}^{(0)}$ are negligibly small compared with their values at the time under consideration, we may omit them in our present analysis.[10]

The relative velocity and separation of the two particles at the present time are then caused by the differences in the forces experienced by the two particles during the history of their flight.

Consider now the results of the statistical analysis involving an ensemble of experiments, each involving a pair of particles. We have a situation very similar to Taylor's one-particle analysis, except that

$$\mathbf{v}(0)=0. \tag{7}$$

For a short time interval t over which the particle velocity has not changed very much, Taylor obtained

$$\mathbf{x}^2=\mathbf{u}^2t^2,$$

while no dispersion will be predicted in the present situation. The particles simply meander together with the large-scale motions.

For longer time intervals, $\mathbf{v}^2$ will be built up according to the law (cf. equation (4))

$$\overline{\mathbf{v}^2} = 2Bt, \quad B = \overline{\mathbf{a}_r^2} \int_0^{\tau_1} R(\tau) d\tau, \tag{8}$$

for $t \gg \tau_1$, where t is the time interval after the release of the particles, and $\mathbf{a}_r$ is the relative acceleration. To calculate the mean square dispersion $\overline{\mathbf{x}^2}$, we note that for $\mathbf{v}(0) = 0$, $\mathbf{x}(0) = 0$, we have

$$\mathbf{x}(t) = \int_0^t (t - t')\mathbf{a}_r(t')dt' \tag{9}$$

and hence

$$\overline{\mathbf{a}_r(t) \cdot \mathbf{x}(t)} = \overline{|\mathbf{a}_r|^2} \int_0^t \tau R(\tau) d\tau, \tag{10}$$

which may be approximated by

$$\overline{\mathbf{a}_r(t) \cdot \mathbf{x}(t)} = B\tau_2 \tag{11}$$

for $t > \tau_1$, where

$$\tau_2 = \int_0^{\tau_1} \tau R(\tau)\, d\tau / \int_0^{\tau_1} R(\tau) d\tau. \tag{12}$$

By combining (8) and (11), we have, for $t \gg \tau_1$,

$$\frac{d^2}{dt^2}\left(\frac{1}{2}\overline{\mathbf{x}^2}\right) = B(2t + \tau_2) \cong 2Bt,$$

since τ_2 is expected to be of the order of τ_1. Thus, the dispersion coefficient is

$$D = \frac{d}{dt}\left(\frac{\overline{\mathbf{x}^2}}{2}\right) = Bt^2 \tag{13}$$

and

$$\overline{\mathbf{x}^2} = \frac{2}{3} Bt^3. \tag{14}$$

Eliminating t between these two equations, we obtain

$$D = \left(\frac{3}{2}\right)^{2/3} \cdot B^{1/3} (\overline{\mathbf{x}^2})^{2/3} \tag{15}$$

which is Richardson's law. Notice that for the strict validity of this law, one must be comparing various situations with the same value of B.

I wish to thank Professor W. Feller for his interest in this problem and for his advice on the general theory of diffusion and Markov processes.

* On leave of absence from Massachusetts Institute of Technology, Cambridge, Massachusetts. The author is grateful to Professor J. R. Oppenheimer for the hospitality extended to him at the Institute for Advanced Study.

[1] Richardson, L. F., *Proc. Roy. Soc.* (*London*), **A110**, 709 (1926).

[2] Kolmogoroff, A. N., *C. R. Acad. Sci. U.R.S.S.*, **30**, 301 (1941). Throughout this note, we shall refer to Kolmogoroff's theory in the "nonviscous form."

[3] Batchelor, G. K., and A. A. Townsend, in *Surveys in Mechanics* (Cambridge University Press, 1956), p. 384.

[4] Taylor, Sir Geoffrey in *Advances in Geophysics* (New York: Academic Press, Inc., 1959), vol. 6, p. 101.

[5] Batchelor, G. K., *ibid.*, p. 449.

[6] Taylor, Sir Geoffrey, *Proc. London Math. Soc.*, **20**, 196 (1921).

[7] Obukhov, A. M., in *Advances in Geophysics* (New York: Academic Press, Inc., 1959), vol. 6, p. 113.

[8] Uhlenbeck, G. E., and L. S. Ornstein, *Phys. Rev.*, **36**, 823 (1930); Chandrasekhar, S., *Rev. Mod. Phys.*, **15**, 1 (1943).

[9] It is also the requirement in Taylor's theory of diffusion by continuous movements. Generally speaking, τ must not be so large that $\int_{\tau_1}^{\tau} R(\tau)\, d\tau$ is no longer negligible compared with $\int_0^{\tau_1} R(\tau)\, d\tau$.

[10] It is not difficult to include $v^{(0)}$ and $r^{(0)}$ in our analysis. The details have been carried out and will be presented in a later communication where other refinements and extensions will be included.

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*UNITARY OPERATOR BASES**

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To qualify as the fundamental quantum variables of a physical system, a set of operators must suffice to construct all possible quantities of that system. Such operators will therefore be identified as the generators of a complete operator basis. Unitary operator bases are the principal subject of this note.[1]

Two state vector space coordinate systems and a rule of correspondence define a unitary operator. Thus, given the two ordered sets of vectors $\langle a^k|, \langle b^k|, k = 1..N$, and their adjoints, we construct

$$U_{ab} = \sum_{k=1}^{N} |a^k\rangle \langle b^k|$$

$$U_{ba} = \sum_{k=1}^{N} |b^k\rangle \langle a^k|$$

which are such that

$$\langle a^k| U_{ab} = \langle b^k|, \; U_{ab}|b^k\rangle = |a^k\rangle$$

$$\langle b^k| U_{ba} = \langle a^k|, \; U_{ba}|a^k\rangle = |b^k\rangle$$

and

$$U_{ab}U_{ba} = U_{ba}U_{ab} = 1, \; U_{ab}\dagger = U_{ba},$$

implying the unitary property

$$U\dagger = U^{-1}$$

for both U_{ab} and U_{ba}. If a third ordered coordinate system is given, $\langle c^k|, k = 1..N$, we can similarly define the unitary operators U_{ac}, U_{bc}, which obey the composition property

$$U_{ab}U_{bc} = U_{ac}.$$

A unitary operator is also implied by two orthonormal operator bases in a given space, that have the same multiplication properties:

$$X(\alpha')X(\alpha'') = \sum_{\alpha=1}^{N^2} X(\alpha)\,\langle\alpha|\,\alpha'\alpha''\rangle$$

$$Y(\alpha')Y(\alpha'') = \sum_{\alpha} Y(\alpha)\,\langle\alpha|\,\alpha'\alpha''\rangle.$$

Let us define

$$U = \lambda \sum_{\alpha} X(\alpha)Y(\alpha)\dagger$$

and observe that

$$X(\alpha')U = \lambda \sum_{\alpha\alpha''} X(\alpha)\,\langle\alpha|\,\alpha'\alpha''\rangle\; Y(\alpha'')\dagger = UY(\alpha'),$$

where the latter statement follows from the remark that the $Y(\alpha)\dagger$ form an orthonormal basis and therefore

$$Y(\alpha)\dagger Y(\alpha') = \sum_{\alpha''} Y(\alpha'')\dagger tr Y(\alpha)\dagger Y(\alpha')Y(\alpha'')$$

$$= \sum_{\alpha''} \langle\alpha|\,\alpha'\alpha''\rangle\; Y(\alpha'')\dagger.$$

We also have the adjoint relation

$$Y(\alpha)\dagger U\dagger = U\dagger X(\alpha)\dagger$$

and in consequence

$$UU\dagger = \lambda\sum X(\alpha)Y(\alpha)\dagger U\dagger$$

$$= \lambda\sum X(\alpha)U\dagger X(\alpha)\dagger = 1\lambda tr U\dagger,$$

according to the completeness of the $X(\alpha)$ basis. Thus the operator U is unitary if we choose

$$\lambda^{-1} = trU\dagger = \lambda^*\sum_{\alpha} \langle X(\alpha)|\,Y(\alpha)\rangle,$$

and, to within the arbitrariness of a phase constant,

$$\lambda = [\sum \langle X(\alpha)|\,Y(\alpha)\rangle]^{-1/2}$$

The converse theorems should be noted. For any unitary operator U, the orthonormal basis

$$Y\,(\alpha) = U^{-1}\,X\,(\alpha)\,U$$

obeys the same multiplication law as the $X(\alpha)$, and the $Y(\alpha)\dagger$ are given by the same linear combination of the $Y(\alpha)$ as are the $X(\alpha)\dagger$ of the $X(\alpha)$ set. In particular, if $X(\alpha)$ is a Hermitian basis, so also is $Y(\alpha)$.

We cannot refrain from illustrating these remarks for the simplest of the N^2-dimensional operator spaces, the quaternion space associated with a physical system possessing only two states. If a particular choice of these is arbitrarily designated as $+$ and $-$, we obtain the four measurement symbols $M(\pm, \pm)$, and can then introduce a Hermitian orthonormal operator basis

$$X(\alpha) = 2^{-1/2}\sigma_\alpha \quad \alpha = 0,..3$$

such that

$$\sigma_0 = 1.$$

Accordingly, the three operators σ_k, $k = 1, 2, 3$, obey

$$tr\ \sigma_k = 0, \quad {}^1/_2 tr\ \sigma_k\sigma_l = \delta_{kl},$$

and an explicit construction is given by

$$\sigma_1 = M(+,-) + M(-,+),\ \sigma_2 = -iM(+,-) + iM(-,+),$$
$$\sigma_3 = M(+,+) - M(-,-),$$

the coefficients of which constitute the well-known Pauli matrices. With these definitions, the multiplication properties of the σ operators can be expressed as

$$\sigma_k\sigma_l = \delta_{kl} + i\sum_m \epsilon_{klm}\sigma_m,$$

or, equivalently, by the additional trace

$${}^1/_2 tr\sigma_k\sigma_l\sigma_m = i\epsilon_{klm}$$

where ϵ_{klm} is the alternating symbol specified by $\epsilon_{123} = +1$. If we now introduce any other Hermitian orthonormal operator basis $Y(\alpha) = 2^{-1/2}\bar{\sigma}_\alpha$, with $\bar{\sigma}_0 = 1$, the resulting three-dimensional orthonormal basis transformation

$$\bar{\sigma}_k = \sum_{l=1}^{3} r_{kl}\sigma_l, \qquad k = 1, 2, 3$$

is real and orthogonal,

$$r^* = r,\ r^T r = 1.$$

The multiplication properties of the σ-basis assert that

$${}^1/_2\ tr\ \bar{\sigma}_k\bar{\sigma}_l\bar{\sigma}_m = i\epsilon_{klm}\ det\ r,$$

where, characteristic of an orthogonal transformation, $det\ r = \pm 1$. If the orthogonal transformation is proper, the multiplication properties of the $\bar{\sigma}$-basis coincide with those of the σ-basis, while with an improper transformation the opposite sign of i is effectively employed in evaluating the $\sigma_k\bar{\sigma}_l$ products. Hence only in the first situation, that of a pure rotation, does a unitary operator exist such that

$$\sum_l r_{kl}\sigma_l = U^{-1}\sigma_k U.$$

The unitary operator is constructed explicitly as[2]

$$U = \lambda^1/_2 \sum_{\alpha=0}^{3} \sigma_\alpha\bar{\sigma}_\alpha$$
$$= \lambda^1/_2[1 + tr\ r + i\sum_{m=1}^{3} r_{kl}\epsilon_{klm}\sigma_m]$$

with

$$\lambda = (1 + tr\ r)^{-1/2}.$$

Let us return to the definition of a unitary operator through the mapping of one coordinate system or another, and remark that the two sets of vectors can be identical, apart from their ordering. Thus, consider the definition of a unitary operator V by the cyclic permutation

$$\langle a^k | V = \langle a^{k+1} |, \; k = 1..N$$

where

$$\langle a^{N+1} | = \langle a^1 |,$$

which indicates the utility of designating the same state by any of the integers that are congruent with respect to the modulus N. The repetition of V defines linearly independent unitary operators,

$$\langle a^k | V^n = \langle a^{k+n} |,$$

until we arrive at

$$\langle a^k | V^N = \langle a^{k+N} | = \langle a^k |.$$

Thus

$$V^N = 1$$

is the minimum equation, the polynomial equation of least degree obeyed by this operator, which we characterize as being of period N. The eigenvalues of V obey the same equation and are given by the N distinct complex numbers

$$v' = e^{\frac{2\pi ik}{N}} = v^k, \qquad k = 0, ..N-1.$$

Unitary operators can be regarded as complex functions of Hermitian operators, and the entire spectral theory of Hermitian operators can be transferred to them. If the unitary operator V has N distinct eigenvalues, its eigenvectors constitute an orthonormal coordinate system. The adjoint of a right eigenvector $|v'\rangle$ is the left eigenvector $\langle v'|$ associated with the same eigenvalue, and the products

$$|v'\rangle \langle v'| = M(v')$$

have all the properties required of measurement symbols. Now let us observe that the factorization of the minimum equation for V that is given by

$$[(V/v') - 1] \sum_{l=0}^{N-1} (V/v')^l = 0$$

permits the identification of the Hermitian operator

$$M(v') = 1/N \sum (v')^{-l} V^l$$

to within the choice of the factor N^{-1}, which is such that

$$\langle v' | M(v') = \langle v' |.$$

On multiplying $M(v^k)$ by $\langle a^N|$ and using the defining property of V, we obtain

$$\langle a^N | v^k \rangle \langle v^k | = 1/N \sum_l \langle a^l | e^{-\frac{2\pi i}{N} kl}$$

from which follows

$$|\langle a^N|v^k\rangle|^2 = 1/N.$$

Then, with a convenient phase convention for $\langle a^N|v^k\rangle$ we get

$$\langle v^k| = N^{-1/2}\sum_l \langle a^l| e^{-\frac{2\pi i}{N}kl}$$

which is also expressed by

$$\langle v^k|a^l\rangle = N^{-1/2}e^{-\frac{2\pi i}{N}kl}, \langle a^l|v^k\rangle = N^{-1/2}e^{\frac{2\pi i}{N}kl},$$

the elements of the transformation functions that connect the given coordinate system with the one supplied by the eigenvectors of the unitary operator that cyclically permutes the vectors of the given system.

Turning to the new coordinate system $\langle v^k|$, we define another unitary operator by the cyclic permutation of this set. It is convenient to introduce U such that

$$\langle v^k|U^{-1} = \langle v^{k+1}|$$

which is equivalent to

$$\langle v^k|U = \langle v^{k-1}|.$$

This operator is also of period N,

$$U^N = 1,$$

and has the same spectrum as V,

$$u' = e^{\frac{2\pi ik}{N}} = u^k, k = 0..N-1.$$

After using the property $U^{N-l} = U^{-l}$ to write the corresponding measurement symbol as

$$M(u') = 1/N\sum_{l=0}^{N-1}(u')^l U^{-l},$$

we follow the previous procedure to construct the eigenvectors of U,

$$\langle u^k| = N^{-1/2}\sum_l \langle v^l| e^{\frac{2\pi i}{N}kl} = \sum_l \langle a^k|v^l\rangle\langle v^l| = \langle a^k|.$$

Thus, the original coordinate system is regained and our results can now be stated as the reciprocal definition of two unitary operators and their eigenvectors,

$$\langle u^k|V = \langle u^{k+1}|$$

$$\langle v^k|U^{-1} = \langle v^{k+1}|.$$

The relation between the two coordinate systems is given by

$$\langle u^k|v^l\rangle = N^{-1/2}e^{\frac{2\pi i}{N}kl}, \langle v^l|u^k\rangle = N^{-1/2}e^{-\frac{2\pi i}{N}kl},$$

and, supplementing, the periodic properties

$$U^N = V^N = 1,$$

we infer from the comparison

$$\langle u^k| UV = \langle u^{k+1}| u^k, \langle u^k| VU = \langle u^{k+1}| u^{k+1} = \langle u^{k+1}| u^k e^{\frac{2\pi i}{N}}$$

that

$$VU = e^{\frac{2\pi i}{N}} UV.$$

As a consequence of the latter result, we also have

$$V^l U^k = e^{\frac{2\pi i}{N} kl} U^k V^l.$$

Each of the unitary operators U and V is a function of a Hermitian operator that in itself forms a complete set of physical properties. It is natural to transfer this identification directly to the unitary operators which are more accessible than the implicit Hermitian operators. Accordingly, we now speak of the statistical relation between the properties U and V, as described by the probability

$$p(u', v'') = |\langle u'| v''\rangle|^2 = 1/N.$$

The significance of this result can be emphasized by considering a measurement sequence that includes a nonselective measurement, as in

$$p(u', v, u'') = \sum_{v'} p(u', v')p(v', u'') = 1/N,$$

for this asserts that the intervening non-selective v-measurement has destroyed all prior knowledge concerning u-states. Thus the properties U and V exhibit the maximum degree of incompatibility. We shall also show that U and V are the generators of a complete orthonormal operator basis, such as the set of N^2 operators

$$X(mn) = N^{-1/2} U^m V^n, \; m, n = 0..N - 1,$$

and therefore together supply the foundation for a full description of a physical system possessing N states. Both of these aspects are implied in speaking of U and V as a *complementary* pair of operators.[3] Incidentally, there is complete symmetry between U and V, as expressed by the invariance of all properties under the substitution

$$U \rightarrow V, \; V \rightarrow U^{-1}.$$

The latter could be emphasized by choosing the elements of the operator basis as

$$N^{-1/2} e^{\frac{\pi i}{N} mn} U^m V^n = N^{-1/2} e^{-\frac{\pi i}{N} mn} V^n U^m,$$

which are invariant under this substitution when combined with $m \rightarrow n$, $n \rightarrow -m$.

One proof of completeness for the operator basis generated by U and V depends upon the following lemma: If an operator commutes with both U and V it is necessarily a multiple of the unit operator. Since U is complete in itself, such an operator must be a function of U. Then, according to the hypothesis of commutativity with V, for each k we have

$$0 = \langle u^k| [f(U)V - Vf(U)]| u^{k+1}\rangle = f(u^k) - f(u^{k+1}),$$

and this function of U assumes the same value for every state, which identifies it with that multiple of the unit operator. Now consider, for arbitrary Y,

$$\sum_{mn} X(mn)YX(mn)\dagger = 1/N \sum_{mn} U^m V^n Y V^{-n} U^{-m} = 1/N \sum_{mn} V^n U^m Y U^{-m} V^{-n},$$

and observe that left and right hand multiplication with U and U^{-1}, respectively, or with V and V^{-1}, only produces a rearrangement of the summations. Accordingly, this operator commutes with U and V. On taking the trace of the resulting equation, the multiple of unity is identified with $tr\ Y$, and we have obtained

$$\sum_{m,\,n\,=\,0}^{N\,-\,1} X(mn)YX(mn)\dagger = 1\ tr\ Y,$$

the statement of completeness for the N^2-dimensional operator basis $X(mn)$. Alternatively, we demonstrate that these N^2 operators are orthonormal by evaluating

$$\langle X(mn) | X(m'n') \rangle = 1/N\ tr\ U^{m'-m} V^{n'-n}$$

$$= \delta(m, m')\delta(n,n'),\ m, n, m', n' = 0..N - 1.$$

The unit value for $m = m'$, $n = n'$ is evident. If $m \neq m'$, the difference $m' - m$ can assume any value between $N - 1$ and $-(N - 1)$, other than zero. When the trace is computed in the v-representation, the operator $U^{m'-m}$ changes each vector $\langle v^k|$ into the orthogonal vector $\langle v^{k+m-m'}|$ and the trace vanishes. Similarly, if $n \neq n'$ and the trace is computed in the u-representation, each vector $\langle u^k|$ is converted by $V^{n'-n}$ into the orthogonal vector $\langle u^{k+n'-n}|$ and the trace equals zero.

One application of the operator completeness property is worthy of attention. We first observe that

$$U^m V^n U V^{-n} U^{-m} = e^{\frac{2\pi i}{N} n} U = u^n U$$

$$U^m V^n V V^{-n} U^{-m} = e^{-\frac{2\pi i}{N} m} V = v^{-m} V,$$

which exhibits the unitary transformations that produce only cyclic spectral translations. Now, if Y is given as an arbitrary function of U and V, the completeness expression of the operator basis reads

$$1/N^2 \sum_{u'v'} F(u'U, v'V) = 1/N\ tr\ F$$

which is a kind of ergodic theorem, for it equates an average over all spectral translations to an average over all states. The explicit reference to operators can be removed if $F(U, V)$ is constructed from terms which, like the individual operators $X(mn)$, are ordered with U standing everywhere to the left of V. Then we can evaluate a matrix element of the operator equation, corresponding to the states $\langle u^0|$ and $|v^0\rangle$, which gives the numerical relation

$$tr\ F(U,V) = 1/N \sum_{u'v'} F(u', v').$$

It is interesting to notice that a number of the powers U^k, $k = 1\ ..\ N - 1$, can have the period N. This will occur whenever the integers k and N have no common factor and thus the multiplicity of such operators equals $\phi(N)$, the number of in-

tegers less than and relatively prime to N. Furthermore, to every such power of U there can be associated a power V^l, also of period N, that obeys with U^k the same operator equation satisfied by U and V,

$$V^l U^k = e^{\frac{2\pi i}{N}} U^k V^l.$$

This requires the relation

$$kl = 1 \ (mod\ N),$$

and the unique solution provided by the Fermat-Euler theorem is

$$l = k^{\phi(N)-1} \ (mod\ N).$$

The pair of operators U^k, V^l also generate the operator basis $X(mn)$, in some permuted order.

We shall now proceed to replace the single pair of complementary operators U, V by several such pairs, the individual members of which have smaller periods than the arbitrary integer N. This leads to a classification of quantum degrees of freedom in relation to the various irreducible, prime periods. Let

$$N = N_1 N_2$$

where the integers N_1 and N_2 are relatively prime, and define

$$U_1 = U^{N_2} \qquad U_2 = U^{N_1}$$
$$V_1 = V^{l_1 N_2} \qquad V_2 = V^{l_2 N_1}$$

with

$$l_1 = N_2^{\phi(N_1)-1} \ (mod\ N_1),\ l_2 = N_1^{\phi(N_2)-1} \ (mod\ N_2).$$

It is seen that U_1, V_1 are of period N_1, while U_2, V_2 have the period N_2, and that the two pairs of operators are mutually commutative, as illustrated by

$$V_1 U_2 = e^{\frac{2\pi i}{N} l_1 N_1 N_2} U_2 V_1 = U_2 V_1.$$

Furthermore,

$$V_1 U_1 = e^{\frac{2\pi i}{N_1}} U_1 V_1$$
$$V_2 U_2 = e^{\frac{2\pi i}{N_2}} U_2 V_2,$$

so that U_1, V_1 and U_2, V_2 constitute two independent pairs of complementary operators associated with the respective periods N_1 and N_2. We also observe that the $N = N_1 N_2$ independent powers of U can be obtained as

$$U_1^{m_1} U_2^{m_2} = U^{(m_1 N_1 + m_2 N_2)} \qquad m_1 = 0..N_1 - 1$$
$$m_2 = 0..N_2 - 1$$

since all of these are distinct powers owing to the relatively prime nature of N_1 and N_2. With a similar treatment for V, we recognize that the members of the orthonormal operator basis are given in some order by

$$X(m_1m_2n_1n_2) = N^{-1/2}U_1^{m_1}\,U_2^{m_2}\,V_1^{n_1}\,V_2^{n_2} = \prod_{j=1}^{2} X(m_jn_j),$$

where

$$X(m_jn_j) = N_j^{-1/2}U_j^{m_j}\,V_j^{n_j},\ m_j,\ n_j = 0..N_j - 1.$$

Another approach to this commutative factorization of the operator basis proceeds through the construction of the eigenvalue index $k = 0..N - 1$ with the aid of a pair of integers,

$$k = k_1N_2 + k_2 \qquad k_1 = 0..N_1 - 1$$
$$k_2 = 0..N_2 - 1.$$

Equivalently, we replace

$$u^k = v^k = e^{\frac{2\pi ik}{N}}$$

by

$$u_1^{k_1} = v_1^{k_1} = e^{\frac{2\pi ik_1}{N_1}}$$
$$u_2^{k_2} = v_2^{k_2} = e^{\frac{2\pi i\,k_2}{N_2}}$$

which gives

$$\langle u^k| = \langle u_1^{k_1}\,u_2^{k_2}|,\ \langle v^k| = \langle v_1^{k_1}\,v_2^{k_2}|.$$

On identifying these vectors as the eigenvectors of sets of two commutative unitary operators, we can define $U_{1,2}$, $V_{1,2}$ by the reciprocal relations

$$\langle u_1^{k_1}\,u_2^{k_2}|\,V_1 = \langle u_1^{k_1+1}\,u_2^{k_2}|,\ \langle u_1^{k_1}\,u_2^{k_2}|\,V_2 = \langle u_1^{k_1}\,u_2^{k_2+1}|$$
$$\langle v_1^{k_1}\,v_2^{k_2}|\,U_1^{-1} = \langle v_1^{k_1+1}\,v_2^{k_2}|,\ \langle v_1^{k_1}\,v_2^{k_2}|\,U_2^{-1} = \langle v_1^{k_1}\,v_2^{k_2+1}|$$

which reproduce the properties

$$U_j^{N_j} = V_j^{N_j} = 1,\ V_j\,U_j = e^{\frac{2\pi i}{N_j}}U_jV_j,$$

together with the commutativity of any two operators carrying different subscripts. The orthonormality of the $N_1^2N_2^2 = N^2$ operators $X(m_1m_2n_1n_2)$ can now be directly verified. Also, by an appropriate extension of the preceeding discussion, we obtain the transformation function

$$\langle u_1^{k_1}\,u_2^{k_2}|\,v_1^{l_1}\,v_2^{l_2}\rangle = \prod_{j=1}^{2}\langle u_j^{k_j}|\,v_j^{l_j}\rangle,$$

with

$$\langle u_j^{k_j}|\,v_j^{l_j}\rangle = N_j^{-1/2}e^{\frac{2\pi i}{N_j}k_j\,l_j}.$$

The continuation of the factorization terminates in

$$N = \prod_{j=1}^{f}\nu_j,$$

where f is the total number of prime factors in N, including repetitions. We call this characteristic property of N the number of degrees of freedom for a system possessing N states. The resulting commutatively factored basis

$$X(mn) = \prod_{j=1}^{f} X(m_j n_j)$$

$$X(m_j n_j) = \nu_j^{-1/2} U_j^{m_j} V_j^{n_j}, \; m_j, n_j = 0..\nu_j - 1$$

is thus constructed from the operator bases individually associated with the f degrees of freedom, and the pair of irreducible complementary quantities of each degree of freedom is classified by the value of the prime integer $\nu = 2, 3, 5, \ldots \infty$. In particular, for $\nu = 2$ the complementary operators U and V are anticommutative and of unit square. Hence, they can be identified with σ_1 and σ_2, for example, and the operator basis is completed by the product $-iUV = \sigma_3$.

The characteristics of a degree of freedom exhibiting an infinite number of states can be investigated by making explicit the Hermitian operators upon which U and V depend,

$$U = e^{iq}, \; V = e^{ip}, \; \epsilon = (2\pi/\nu)^{1/2},$$

and where

$$q^k = p^k = k\epsilon, \quad k = 0, \pm 1, \pm 2, .. \pm {}^1/_2(\nu - 1).$$

We shall not carry out the necessary operations for $\nu \to \infty$, which evidently yield the well-known pair of complementary properties with continuous spectra. One remark must be made, however. In this approach one does not encounter the somewhat awkward situation in which the introduction of continuous spectra requires the construction of a new formalism, be it expressed in the language of Dirac's delta function, or of distributions. Rather, we are presented with the direct problem of finding the nature of the subspaces of physically meaningful states and operators for which the limit $\nu \to \infty$ can be performed uniformly.

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[1] For the notation and concepts used here see these PROCEEDINGS, **45**, 1542 (1959), and **46**, 257 (1960).

[2] The absence in the available literature of an explicit statement of this simple, general result is rather surprising. The inverse calculation giving the three-dimensional rotation matrix in terms of the elements of the unitary matrix is very well known (rotation parametrizations of Euler, Cayley-Klein), and the construction of the unitary matrix with the aid of Eulerian angles is also quite familiar.

[3] Operators having the algebraic properties of U and V have long been known from the work of Weyl, H., *Theory of Groups and Quantum Mechanics* (New York: E. P. Dutton Co., 1932), chap. 4, sect. 14, but what has been lacking is an appreciation of these operators as generators of a complete operator basis for any N, and of their optimum incompatibility, as summarized in the attribute of complementarity. Nor has it been clearly recognized that an *a priori* classification of all possible types of physical degrees of freedom emerges from these considerations.

*ESTRADIOL-17β, ESTRONE, AND PROGESTERONE IN THE OVARIES OF DOGFISH (SQUALUS SUCKLEYI)**

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Although ovarian extracts having estrogenic activity have been reported for representatives of all classes of vertebrates and certain invertebrates, little is known of the chemical nature of the active agents in any instance with the exception of mammals. Evidence based on countercurrent partitioning, chromatography, and bioassay methods has been presented for the presence of estradiol-17β in ovarian eggs of the dogfish (*Squalus suckleyi*)[1] and similar procedures have been employed for identifying estradiol-17β, estrone, and estriol obtained from the ovaries of laying hens[2] and the lungfish (*Protopterus annectens*).[3] The present paper presents a more detailed study of the steroid hormones of the ovary of the dogfish (*S. suckleyi*).

Procedures and Results.—One third of the total acetone extracts (2100 ml) from 7.9 Kg of whole ovaries of nonpregnant dogfish (*S. suckleyi*) was evaporated to dryness by a gentle stream of warm air passing over the solution which was contained in a large evaporating dish. The residue was then taken up in ethyl ether and the resulting solution kept at 5°C for a week. At this time a heavy precipitate was found and the supernatant ether layer was carefully decanted. The ether solution was evaporated to an oily residue *in vacuo*. The oil was dissolved in a sufficient volume of 70 per cent ethanol and extracted three times with ligroin (b.p. 30–60°C).

The ethanolic layer of the above fractionation was diluted with water to contain 35 per cent of alcohol. After standing in the cold room (4°C) overnight, three layers were formed. The lowest of these, the alcoholic layer, was removed and washed three times with ligroin. The washings were combined with the two upper layers, and backwashed with 35 per cent ethanol. A sample from the pooled alcoholic extracts was bioassayed and gave positive results for estrogenic activity.

The 35 per cent alcoholic extract was evaporated to dryness and the residue partitioned with 29 transfers between 70 per cent ethanol and 50 per cent chloroform–50 per cent carbon tetrachloride. Five fractions were made by combining the contents of tubes 0–2, 3–9, 10–19, 20–25, and 26–29. Each fraction was taken to dryness *in vacuo* and the residue dissolved in 10 ml of methanol. A 1.0 ml portion of each fraction was used in the preparation of serial dilutions for bioassay by the Astwood[4] method. In the final tests, the results for which are shown in Table 1, each animal received extract equivalent to 1.8 grams of fresh ovarian tissue in 0.1 ml of sesame oil. Estrogenic activity was found only in the fractions from tubes 3–9 and 10–19. Estimates based on the activity in tubes 10–19, later identified as estradiol-17β, indicated that the whole extract (7.9 Kg) contained at least 948 μg of estradiol or 120 μg per kilogram of fresh ovarian tissue.

The material from tubes 10–19 was evaporated to dryness and the gummy residue dissolved in 30 ml of benzene. This solution was applied to an alumina

TABLE 1

BIO-ASSAY OF FRACTIONS OBTAINED BY PARTITIONING 35 PER CENT ALCOHOLIC EXTRACT BETWEEN 70 PER CENT ETHANOL AND A 50-50 PER CENT MIXTURE OF CHLOROFORM AND CARBON TETRACHLORIDE

Fraction	Average Uterine Wet Weight	Per Cent Increase over Control
Control	21.8 (25)*	..
0–2	21.2 (4)	0
3–9	28.9 (4)	32.6
10–19	40.8 (4)	87.1
20–25	20.9 (4)	0
26–29	21.9 (4)	0

* Number of 22-day old rats, weighing 55–60 grams, used in each assay group.

column (Woelm alumina activity grade I). The column was eluted with ligroin (200 ml), benzene (100 ml), ethyl acetate (250 ml) and chloroform (200 ml). Each fraction was bioassayed and estrogenic activity was found only in the chloroform eluate. After evaporation of the solvent approximately 2 mg of a yellow, oily substance remained.

The oily residue from the chloroform eluate was again dissolved in a minimal quantity of benzene and applied to another alumina column. This column was

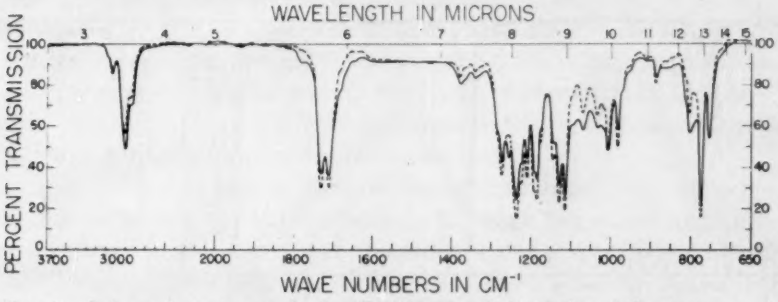

FIG. 1.—Infra-red spectrograph of authentic extradiol-17β dinaphthoate (———) and experimental estradiol-17β dinaphthoate (- - - - - - -).

eluted with benzene containing increasing amounts of methanol. The sixth 100 ml fraction (5 per cent methanol) on evaporation yielded a small residue of white crystals in oil. The semicrystalline material was re-precipitated from aqueous methanol yielding 0.88 mg of an amorphous substance melting from 148–155°C. This was reacted with 1-naphthoyl-chloride as described by MacCorquodale, Thayer, and Doisy.[5] The derivative was recrystallized from ethanol, yielding 1.03 mg of white crystals melting from 192–197°C. A mixed melting point with authentic estradiol dinaphthoate (mp 197°C) showed no depression. The remainder of the material was dissolved in carbon disulfide and an infrared spectrum was recorded on a Perkin-Elmer model 21 double beam spectrophotometer. This was compared with a spectrum of approximately 1 mg of authentic estradiol dinaphthoate and the two spectra were found to be identical (Fig. 1).

The dark brown residue obtained on combining the contents of tubes 3–9 from the first countercurrent distribution was dissolved in 50 ml of 1 *N* sodium hydroxide and washed three times with 25 ml portions of ether. The alkaline residue was partially neutralized to pH 7.5 and again extracted with three 50 ml portions of ether. This last extract, now straw-yellow in color, was taken to dryness *in vacuo*.

Since it was uncertain whether the estrogenic activity remaining in this portion of the extract was due to tailing of the estradiol peak in the countercurrent partition or to the presence of estrone, this residue was chromatographed on paper in a toluene-propylene glycol system.[6] After development of the chromatogram for 16 hours the areas corresponding to estradiol and estrone, as determined by comparison with a simultaneously developed control strip, were eluted and a portion thereof was bioassayed.

The substance eluted near the starting line and corresponding to estradiol produced 56 per cent average increase in uterine wet weight when a $^1/_{50}$ aliquot was divided equally and given to two rats. This portion representing estradiol tailing in the first partition was not further investigated.

The material 36–39 cm from the origin and corresponding in travel rate to authentic estrone was eluted from the strip and a $^1/_{50}$ portion caused a 66 per cent average increase in uterine wet weight when tested on two rats. This material was further purified by formation of the Girard T complex, washing the water soluble phase with ether followed by acid hydrolysis and reextraction of the phenolic material

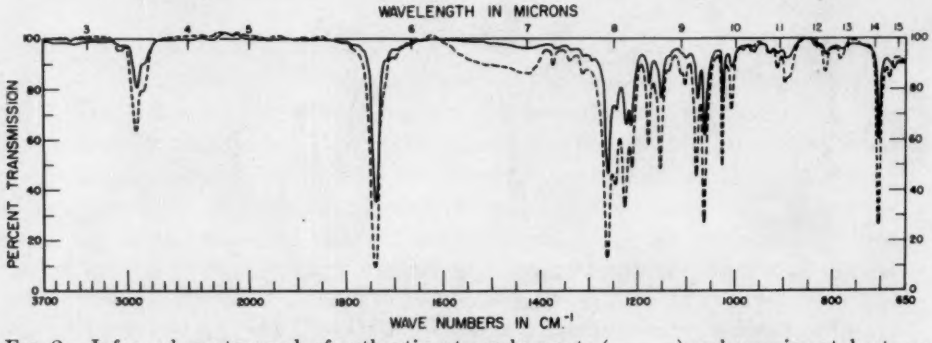

FIG. 2.—Infra-red spectrograph of authentic estrone benzoate (———) and experimental estrone benzoate (-------).

with ether. The amorphous white residue was then reacted with benzoyl chloride in pyridine at 0°C. After leaving the mixture overnight it was poured over cracked ice and extracted with ether. The ether layer was then washed successively with dilute hydrochloric acid, 10 per cent sodium bicarbonate solution and water. Following these washings the solution was taken to dryness *in vacuo*. The dried residue was applied in 2 ml of benzene to a 10 × 50 mm alumina column (Woelm, neutral, alumina activity grade II). The column was eluted with 75 ml of ligroin followed by 200 ml of benzene. The eluates were collected in 25 ml portions and the ninth eluate after evaporation left a white semicrystalline residue. This material was dissolved in a few drops of carbon disulfide and an infrared absorption spectrum was recorded. This spectrum was identical with one obtained from authentic estrone benzoate (Fig. 2).

The ligroin fractions were combined and subjected to procedures for the isolation of progestational substances. They were evaporated to an oily black residue and then applied to an alumina column (15 × 250 mm) in benzene. The column was eluted with petroleum ether, benzene, chloroform, and alcohol. A total of eleven 100 ml fractions were collected and individually taken to dryness *in vacuo* and an

aliquot ($^1/_{20}$) of each was tested for progestational activity by the method of Hooker and Forbes.[7] A positive reaction was given by fractions five, seven, eight, nine, and eleven. These fractions of the crude material were combined in two lots, four through seven in one (A) and eight through eleven in the other (B) and each applied to an alumina column (100 g) in benzene. A control column of 100 g of alumina was developed in like fashion after adsorption of 1.0 mg progesterone and 400 μg each of 20α and 20β-hydroxy-pregn-4-en-3-one. Identification of the control substances after chromatography was carried out by infrared spectroscopy.

The portions of both experimental chromatograms that showed progestational activity and correspond to authentic progesterone were combined and dissolved in 0.5 ml of benzene. This solution was applied to a 2 cm paper strip and the chromatogram was developed in a ligroin, propylene glycol system for $3^3/_4$ hours. A control strip containing progesterone was run simultaneously. On staining the dried control strip with Zimmermann's reagent the color appeared 13–16 cm from the origin. The chromatogram containing the extract was cut and the area from 13–16 cm was eluted with several portions of methanol. The material obtained from the eluate, when bioassayed by the Hooker-Forbes method, gave positive reactions thus further indicating the presence of progesterone.

Conclusions.—The results obtained in this study show conclusively that estradiol-17β and estrone are present in the ovaries of the dogfish, *Squalus suckleyi*. They also support previous evidence[1] for the presence of estradiol-17β in the ova of this species. Estriol was not identified and it seems that in this respect the dogfish may differ from the lungfish, *Protopterus annectens*.[3]

That progesterone was also isolated is strongly indicated but the evidence is not as conclusive as that for the estrogens. Although the chromatographic data and bioassays were in full agreement the amount of material was not sufficient for a more thorough identification. There also were indications of other progestational substances, particularly 20α-hydroxy-pregn-4-en-3-one.

The presence of the same steroid hormones in the ovaries of an elasmobranch fish and mammals suggests that they may be found in all vertebrates and at the same time raises questions regarding function and possible significance in the evolutionary development of the ovary.

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